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# **THERAPEUTIC DNA VACCINATION IN FOLLICULAR LYMPHOMA**

A dissertation submitted to the Open University by

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In candidature for the degree of Doctor of Philosophy

Addenbrooke's NHS Trust

(Sponsoring Establishment)

&

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(Collaborating Establishment)

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## **Declaration**

The work described in this thesis was carried out under the supervision of Dr Willem H Ouwehand and Dr Ian J Harmer in the Department of Haematology, University of Cambridge, between January 2000 and December 2005. It was carried out entirely by me unless otherwise stated in the text. The thesis has not been submitted, either wholly or in part, for a degree, diploma or other qualification at any other university.

Nicola Jennings

December 2005

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## Abbreviations

|       |                                    |
|-------|------------------------------------|
| APC   | Antigen Presenting Cell            |
| APS   | Ammonium persulphate               |
| BCA   | Bicinchoninic Acid                 |
| BSA   | Bovine serum albumin               |
| CaM   | Calmodulin                         |
| CD    | Cluster of differentiation         |
| cDNA  | complementary DNA                  |
| CDR   | Complementarity determining region |
| CHO   | Chinese Hamster Ovary              |
| CH/L  | Constant heavy/light               |
| D1D2  | Domains 1 and 2                    |
| DNA   | Deoxyribo nucleic acid             |
| ECL   | Enhanced chemiluminescence         |
| EDTA  | Ethylene diamine tetra acetic acid |
| ELISA | Enzyme linked immunosorbent assay  |
| FCS   | Foetal Calf Serum                  |
| FR    | Framework                          |
| FrC   | Fragment C                         |
| g     | times gravity                      |
| GP    | Glycoprotein                       |
| His   | Histidine                          |
| HRP   | Horse radish peroxidase            |
| Id    | Idiotypic                          |

|          |   |
|----------|---|
| Ig       | Immunoglobulin  |
| im       | Intra-muscular  |
| IMAC     | Immobilised metal chelate affinity chromatography         |
| kDa      | Kilo-dalton   |
| MCS      | Multiple cloning site                                     |
| MFI      | Median Fluorescent Intensity                              |
| MHC      | Major histocompatibility complex                          |
| min      | Minute  |
| mRNA     | Messenger RNA   |
| NBS      | National Blood Service                                    |
| NIBSC    | National Institute Biological Standards and Control       |
| OD       | Optical Density   |
| O/N      | Overnight   |
| PBS      | Phosphate buffered saline                                 |
| PCR      | Polymerase chain reaction                                 |
| PIFT     | Platelet immunofluorescence test                          |
| rpm      | Revolutions per minute                                    |
| RNA      | Ribonucleic acid  |
| RT       | Room temperature  |
| RT-PCR   | Reverse transcription-polymerase chain reaction           |
| S2       | Schneider 2   |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrlamide gel electrophoresis |
| sec      | Second  |
| TBE      | Tris Borate EDTA  |
| TBS      | Tris buffered saline                                      |

|       |                                      |
|-------|--------------------------------------|
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| TMB   | 3,3',5,5'-Tetramethylbenzidine       |
| TT    | Tetanus toxoid                       |
| VH/L  | Variable heavy/light                 |

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## Abstract

The experiments described in the chapters of this thesis were part of a collaborative project with colleagues at the Universities of Bournemouth, Manchester and Southampton. This Cancer Research-UK and National Blood Service (NBS) sponsored clinical trial reports on one of the first DNA vaccine trials in humans in patients with low-grade follicular lymphoma. Twenty-five patients were enrolled and the V genes encoding the V domains of their surface immunoglobulin (sIg) on the dominant malignant clone were isolated from a lymph node biopsy and single chain variable domain antibody fragments (scFv) were constructed. The V gene cassettes were cloned into the vaccine plasmid, pVAC2. pVAC2 fuses the scFv gene cassette in open reading frame to the gene for fragment C of tetanus toxoid. Each patient vaccine was produced to GMP standards and subjected to pre-release quality and sterility controls. In parallel with the clinical trial all the vaccines were administered to Dutch rabbits and antibody responses were measured. To measure anti-idiotypic responses an inducible/secretable *Drosophila* expression system was modified and calmodulin (CaM) tagged scFv was produced. The scFv-CaM fusion proteins were then used to measure the anti-idiotypic responses in the patients pre- and post-vaccination. The variable domains from another FL patient (but not enrolled in the trial) with an IgM paraprotein and suffering from severe autoimmune thrombocytopenia, were isolated, the scFv constructed, a DNA vaccine prepared and idiotypic antibodies raised. These reagents were used to determine whether the IgM paraprotein produced by the dominant Lymphoma clone was exhibiting anti-platelet reactivity.

## **Chapter 1 – Introduction**

### **1.1 Overview**

Cancer is the generic name for a class of diseases characterised by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis).

Cancer can affect all the cells of the body and each cancer has its own signature. There is no single cause of cancer although certain environmental agents and genetic predispositions can increase a persons propensity to acquire the disease. Treatment for cancer is usually a combination of chemo- and radiotherapy to attack the fast growing malignant cells. However, there is no distinction between malignant cells and healthy fast growing cells in the hair follicles and gastrointestinal (GI) tract. Therapies that target only the cancerous cells are more appealing although this approach requires that the malignant cells express a unique marker that can be preferentially targeted.

This thesis focuses on Follicular Lymphoma (FL), one of the many cancers affecting the cells of the blood and bone marrow. FL is a B cell malignancy that occurs after the B-cell has been exposed to antigen during its development (Figure 1.1). The malignant B cell expresses surface immunoglobulin (sIg) that confers a unique set of antigenic determinants, the idiotype. This idiotype is unique to the malignant B cells and can therefore be used as a target for immunotherapy. Together with colleagues in Southampton, Bournemouth and Manchester a DNA vaccine encoding the amino acid sequence of the idiotype from patients with FL was developed and administered to the patients in a phase I/II clinical trial, the first of its kind in cancer patients.



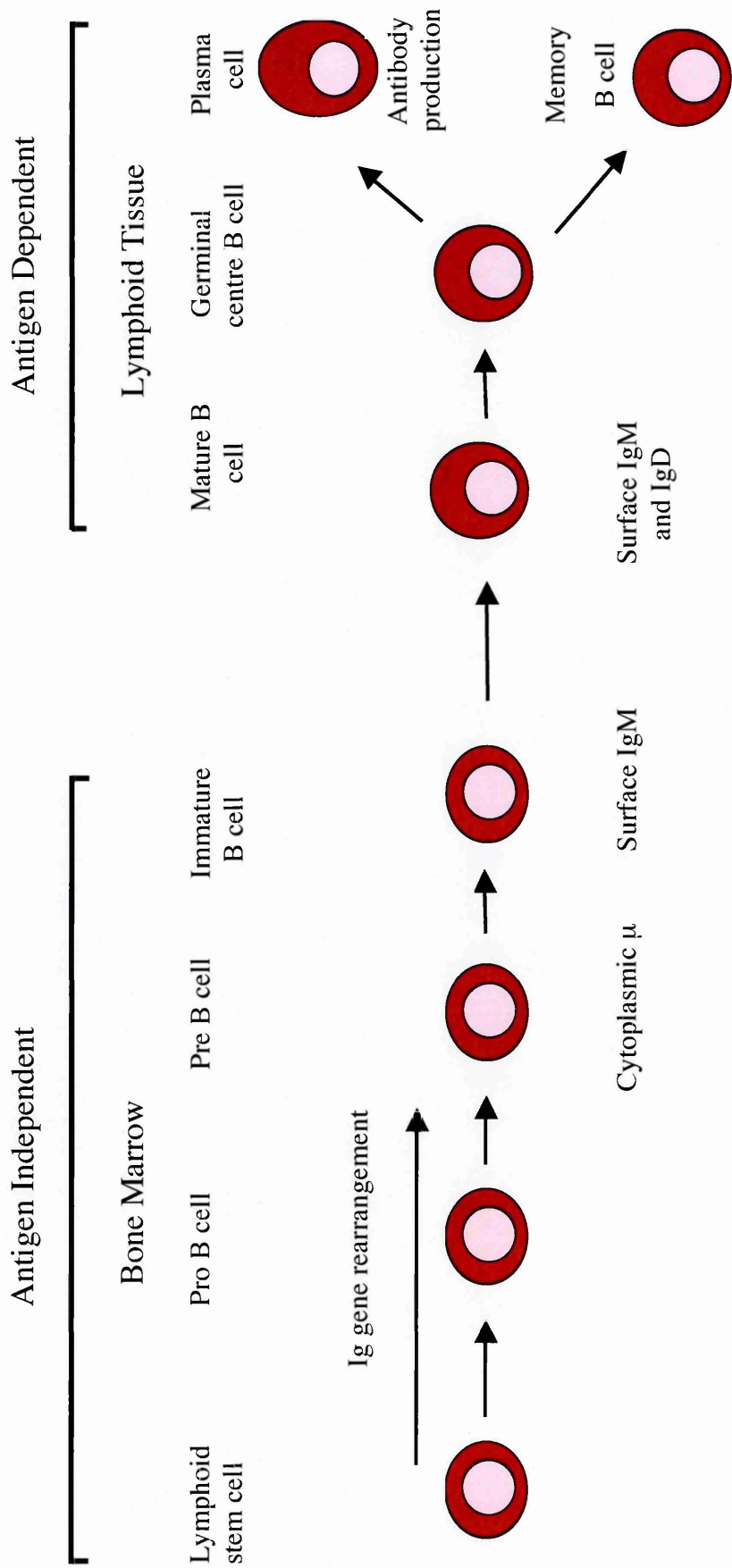


Figure 1.1 Schematic representation of B cell development. The antigen independent stages occur in the bone marrow and the antigen dependent stages occur in the lymphoid tissues. The germinal centre B cell either differentiates into antibody secreting plasma cells or memory B cells. Follicular lymphoma occurs during antigen-dependent B-cell development, in the germinal centre (GC) B cell (right panel).

## 1.2 Immune system

All immune responses involve two steps, firstly, recognition of the pathogen and secondly, a reaction to eliminate it. Immune responses fall into two categories, innate and adaptive. The communication and collaboration between the cells of the immune system is tightly regulated and can be achieved either by direct interaction of surface receptors and ligands, or by secretion of soluble mediators specific for receptors expressed on certain classes of immune cells. This defends the body against non-self.

The innate immune system is the first line of defence, it is not antigen specific and it relies on the body's natural barriers and secretions to eliminate microbes and prevent infections. Elements of the innate system are anatomical barriers, secretory molecules and cellular components. There are three cell types involved in innate immune responses. Firstly, the phagocytic cells (neutrophils, monocytes, and macrophages). Secondly, cells that release inflammatory mediators (basophils, mast cells, and eosinophils) and finally natural killer (NK) cells.

Adaptive, or acquired, immunity refers to an antigen-specific defence mechanism that takes several days to become protective and is specific for a particular pathogen.

Adaptive immunity is a constant process that develops throughout life and generally improves upon repeated exposure to a given infection. The main components of the adaptive immune system are the professional antigen presenting cells (APC) and the B and T lymphocytes (B and T cells) (so called because they were originally discovered in the Bursa of Fabricius and differentiate in the adult bone marrow and thymus respectively) (Miller & Mitchell, 1967; Tonegawa, 1983). B and T cells will recognise the same antigen in different forms, by virtue of their respective antigen specific

receptors, sIg or B cell receptor (BcR) and T cell receptor TcR, expressed on the cell surface (Figure 1.2). Naive B cells are genetically programmed to encode a sIg. The population of polyclonal B cells expresses a vast repertoire of sIg with an estimated diversity of  $10^{12}$  different specificities. Activation and subsequent proliferation of B cells by antigen causes clonal proliferation and selection. During clonal selection the affinity of sIg for the antigen increases by somatic hypermutation (SHM) of the rearranged V genes. Mature antigen selected B cells may differentiate into plasma cells and secrete large amounts of specific soluble Ig (Schebesta *et al.*, 2002). Some will differentiate into memory B cells allowing a more rapid and vigorous immune response when re-challenged.

T cells recognise antigens only when presented on the surface of other cells in the context of the major histocompatibility complex (MHC). The TcR recognises short oligo peptides (9-12 amino acids) derived from the antigen presented in the context of one of two specialised protein complexes - MHC class I and II. The former is expressed on the surface of all somatic cells (except red blood cells) and the latter on specialised cells of the immune system such as antigen presenting cells (APCs), B cells and activated T cells (Roitt *et al.*, 2001). Subsets of T cells have different functions dependant on whether they bind MHC class I (CD8 positive T cells) Cytotoxic T lymphocytes (CTL), responsible for the destruction of host cells that have become infected by intracellular pathogens, or class II (CD4 positive T cells). CD4<sup>+</sup> T cells can be further subdivided into type-1 (T<sub>H</sub>1) and type-2 (T<sub>H</sub>2) helper T cells on the basis of their cytokine profiles. T<sub>H</sub>1 cells promote cell-mediated inflammatory reactions and T<sub>H</sub>2 cells promote strong antibody and allergic responses.

The complex nature of the immune system is typified by the integration of multiple signals that result in either activation or inhibition of a response. A two-step model (Bretscher & Cohn, 1970; Jenkins & Schwartz, 1987), where a positive result will only occur if two activation signals (antigen specific and generic) are received, is required for T cell and full B cell activation and clonal expansion. This introduces a greater degree of control over clonal proliferation with the consequences of antigen recognition and antigen elimination. In the early phase of the response against exogenous antigen professional APC educate T cells, which in turn interact with B cells and in the later phase B cells may become the preferred cells for antigen presentation (Figure 1.2). The cooperation between the different cell types of the immune system and the resulting degree of regulatory complexity is a pre-requisite to maintain tolerance for self, whilst retaining the capacity to achieve effective immune responses against non-self.

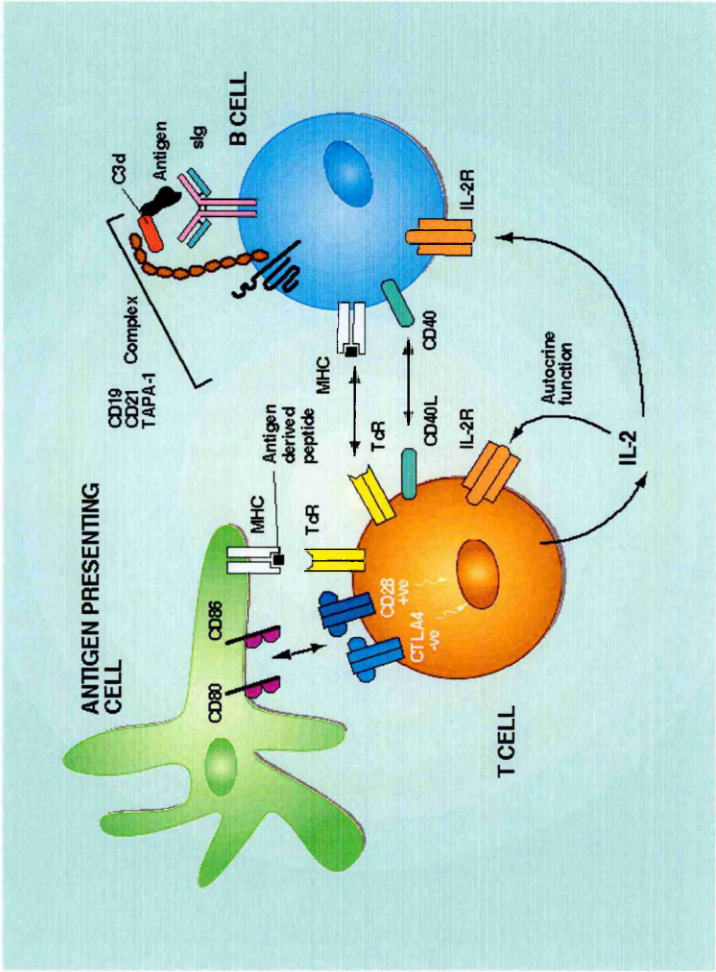


Figure 1.2 Diagrammatic representations of the cells of the adaptive immune system. Surface receptors and ligand illustrating the interaction between an antigen presenting cell (APC), T cell and B cell. T and B cells recognise different epitopes of the same antigen; processed and presented to T cells by APCs or directly recognised by B cells. Secondary signals are required for full activation of both B and T cells. For the T cell CD28 interacts with CD80/86 a process that is regulated by CTLA4 expressed only on activated T cells. For the naive B cell the activation threshold for proliferation can be lowered by concomitant interaction of complement fragment C3d with CD21 and surface Immunoglobulin (slg) with antigen, or via T-B cell interaction through CD40L-CD40. CD – cluster of differentiation, MHC – major histocompatibility complex, IL-2 – interleukin-2, R – receptor, TcR – T cell receptor. Reproduced from the thesis of Erica Wilson with kind permission, University of Cambridge.

### 1.3 B cell development

B cells differentiate from lymphoid stem cells and originate in the bone marrow. There are two main stages of B cell development. The initial development is largely independent of T cells and antigen (Figure 1.1).

The earliest stage of B-cell development has been identified as the progenitor B (pro-B) cell stage, where the Ig genes are in germline configuration. Pro-B cells can be divided into three groups (early, intermediate and late) based on the differential expression of the intranuclear enzyme, terminal deoxynucleotidyltransferase (TdT) and/or the marker B220, a high molecular weight (220 kDa) isoform of CD45R (reviewed by Roitt (Roitt *et al.*, 2001)). As the cells progress through the pro-B stage they rearrange their immunoglobulin (Ig) heavy chain genes and begin to express CD43, CD19, recombination activating gene (RAG)-1 and RAG-2. The late pro-B cells become precursor B (pre-B) cells, during this stage they downregulate TdT, RAG-1, RAG-2 and CD43. Pre-B cells are divided into large mitotically active and small non-dividing pre-B cells. Both express the Ig  $\mu$  heavy chains in the cytoplasm (c $\mu$ ) and the pre-B receptor complex on their surface. Large pre-B cells have successfully rearranged their Ig heavy chain genes and as they differentiate into small pre-B cells they begin to rearrange their Ig light chain genes and upregulate RAG-1 and RAG-2. Finally they differentiate into the immature B cell, which has successfully rearranged light chain genes and expresses surface IgM. Immature B cells develop into mature B cells and begin to express both IgM and IgD on their surface. Mature B cells can exit the bone marrow and migrate into the periphery.

Upon exposure to antigen, specific B cells enlarge and express new cell surface molecules. The enlarged B cells can be stimulated to enter the cell cycle and

differentiate and mature into plasma cells. These cells do not divide and secrete large amounts of antibody. Following the removal of antigen, B cells stop dividing and differentiate into memory B cells. B cells that fail to encounter antigen are removed from the repertoire within a few weeks either by apoptosis or by becoming anergised.

## **1.4 Immunoglobulins**

Immunoglobulins are glycoproteins in the immunoglobulin superfamily that are found both secreted in the blood and tissue fluids of all mammals (antibodies) and carried on the surface of B cells, where they act as specific receptors for antigen (sIg). The terms antibody and immunoglobulin are often used interchangeably. One portion of the antibody molecule binds antigen whilst the other has effector functions.

### **1.4.1 Immunoglobulin structure**

There are five classes of antibody IgG, IgA, IgM, IgD and IgE; they differ in size, charge, amino acid composition and carbohydrate content. Most B cells express two classes of sIg known as IgM and IgD (Melchers *et al.*, 2000).

The basic structure of all immunoglobulins is a unit consisting of two identical heavy (H) chains and two identical light (L) chains of approximately 55 and 24 kDa respectively. The H chain is divided into four domains, one amino terminal variable (VH) domain and three constant (C) domains CH1, CH2 and CH3 (or four in the case of IgM and IgE). The light chain is divided into two domains VL (N-terminal) and CL. The VH and VL domains are linked to the CH1 and CL domains respectively and CH1 is linked to CH2 and CH3 by the hinge region (Figure 1.3).

Two or more disulphide bonds in the hinge region unite the two heavy chains and a disulphide bond couples the CH1 and CL domains. Strong non-covalent attractions bring together the N terminal VH and VL domains to form the antigen recognition site, these bonds form the classic Y-shape of the antibody molecule.

The Ig molecule can be divided into three fragments by digestion with papain (Figure 1.3); two representing the antigen binding V domains with heavy and light chain constant domains CH1/CL (Fab), and one of only the CH2 and CH3 domains (Fc); or after digestion with pepsin a F(ab')<sub>2</sub> fragment composed of both antigen binding domains joined by disulphide bonds, and smaller fragments of the constant domains (reviewed by Roitt (Roitt *et al.*, 2001)). Each of these separate domains has a similar structure known as the immunoglobulin fold, consisting of seven (C domain) or nine (V domains)  $\beta$  strands arranged so that four strands form one  $\beta$  sheet, and three or five strands form a second  $\beta$  sheet (Figure 1.4). Each of the two V domains has three surface exposed hairpin loops connecting the  $\beta$  strands, also known as hypervariable loops or complementarity determining regions (CDR). These six surface exposed loops (three from each VH and VL) form contacts with antigen.



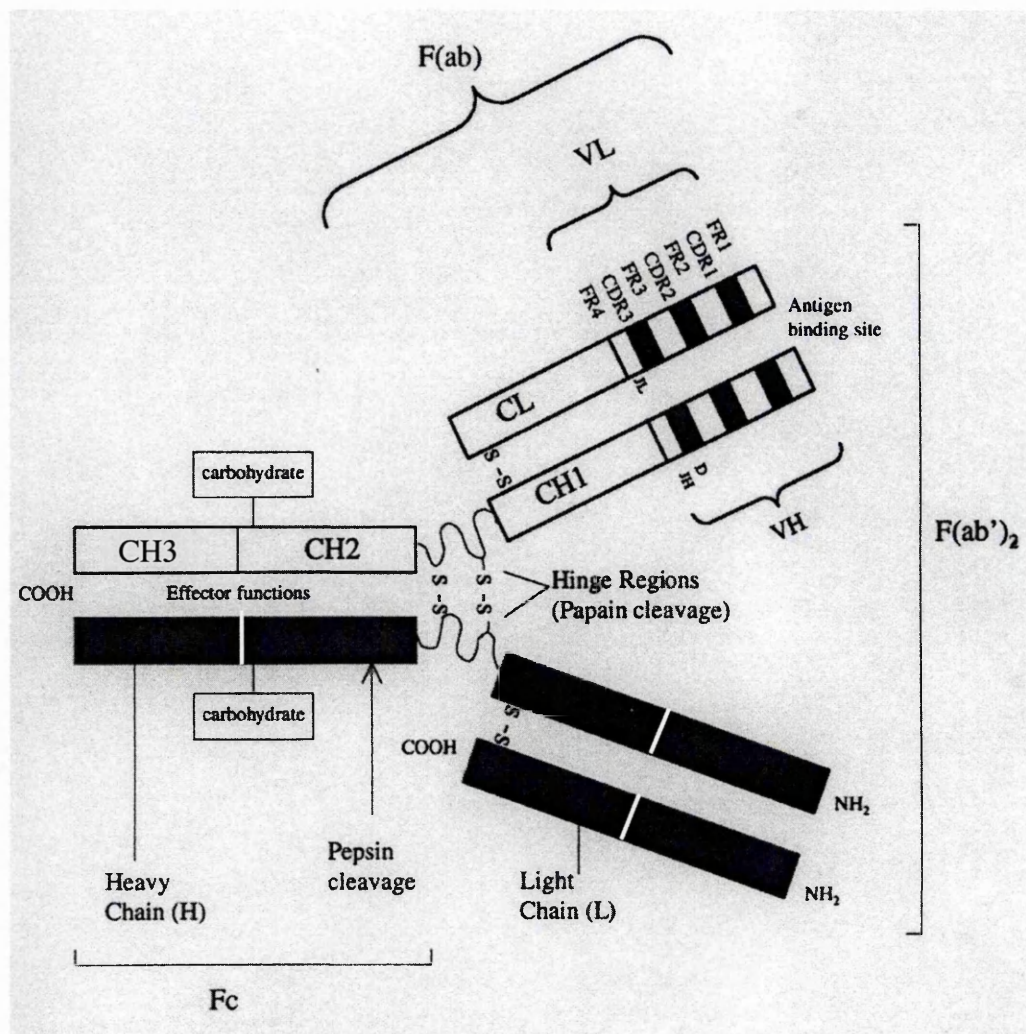


Figure 1.3 A schematic representation of an antibody of the IgG isotype. IgG is composed of two pairs of identical light and heavy polypeptide chains composed of Constant (C) Heavy (H)1, CH2, CH3 or C light (L) domains and Variable (V) H or VL domains. The structure is stabilised by intra- and inter-chain disulphide bonds. Pepsin and papain cleavage sites produce Fc, F(ab) or F(ab)<sub>2</sub> products as. Antigen is bound at the antigen binding sites formed by the association of the VH and VL domains at the amino terminus of the polypeptide chain. (Adapted from the thesis of Darren Hughes with kind permission, University of Cambridge).

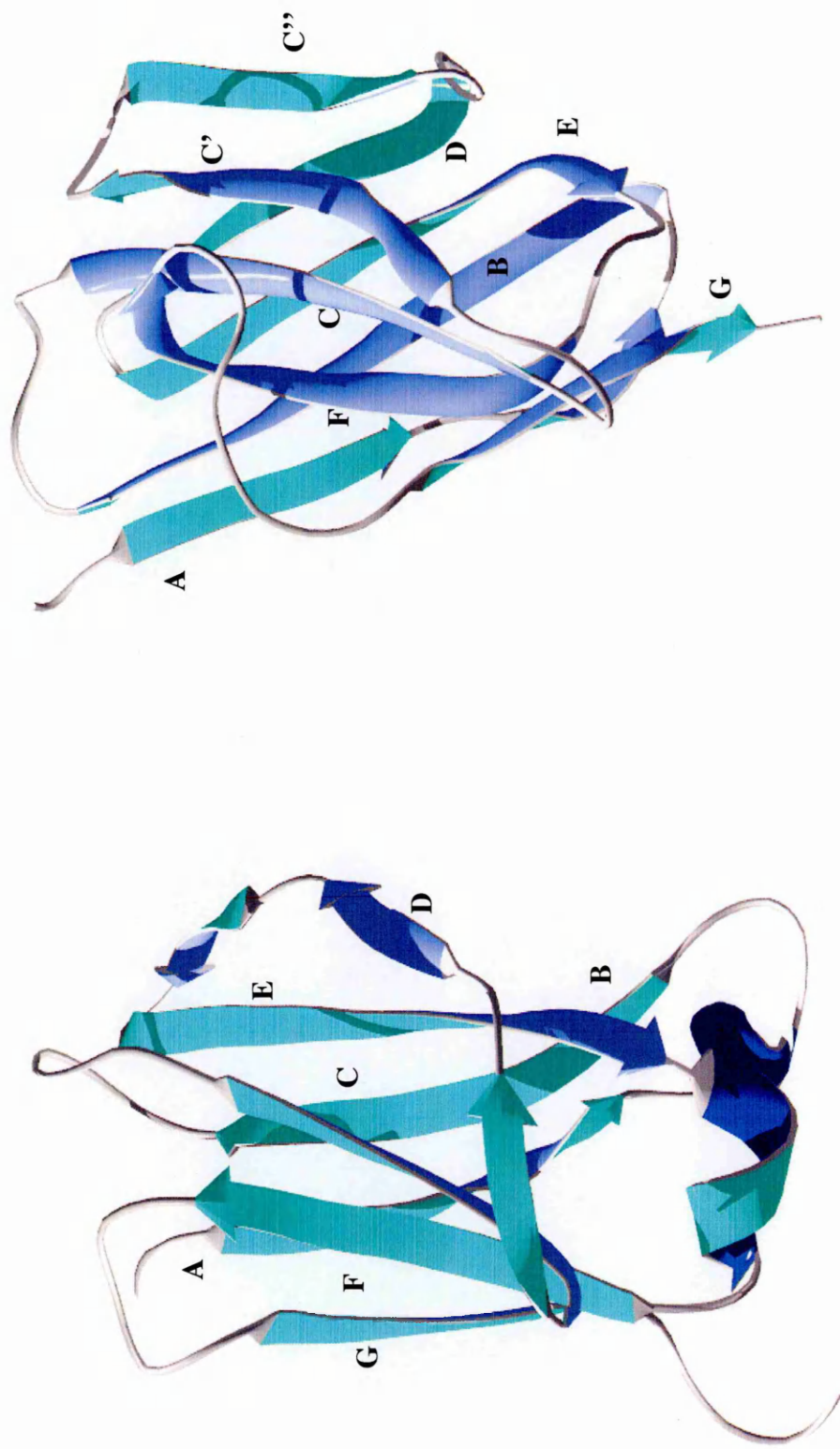


Figure 1.4 Crystal structures of the first constant domain of the heavy (left) and variable domain of the heavy chain (right) from a Human IgG molecule (Idusogie *et al.*, 2000) and a human  $V_H$  domain (Gaur, 2005) respectively. The Immunoglobulin fold is composed of two anti-parallel  $\beta$ -sheets incorporating strands A, B, E and D (left) and C, F, G (plus C' and C'' for the V domain)(right).

### 1.4.2 Immunoglobulin classification

Structural differences between immunoglobulins are used for their classification.

There are three classes of immunoglobulin, which can also be described as immunoglobulin variability.

Firstly, the type of heavy chain an immunoglobulin possesses determines isotypic variation. There are five heavy chains ( $\mu$ ,  $\delta$ ,  $\gamma$ ,  $\epsilon$ ,  $\alpha$ ) giving rise to the nine human isotypes IgM, IgD, IgG 1, 2, 3 and 4, IgE and IgA 1 and 2 respectively (Walter *et al.*, 1991). Secondly, allotypic variation is a genetic difference between individuals within a species, involving different alleles at a given locus. Immunoglobulin allotypes occur mostly as variants of heavy chain constant regions. Finally, idiotypic variation refers to the sequence diversity at the antigen binding site, particularly in the hypervariable regions. The antigen binding site itself is also an antigenic determinant and the collection of epitopes that it produces is known as the idio type (Slater *et al.*, 1955). Idiotypes are usually specific for individual B-cell clones (private idiotypes), but are sometimes shared between different B-cell clones (public or cross-reactive idiotypes) (Roitt *et al.*, 2001).

### 1.4.3 Immunoglobulin function

The primary function of an antibody is to bind antigen. Antibodies have a variety of ways to eliminate the antigen that elicited their production. Some functions are independent of the isotype and reflect the antigen binding capacity of the molecule as defined by its idio type, for example by neutralizing bacterial toxin or by preventing viral attachment to host cells. Other functions are dependent upon the isotype and are

mediated by the constant regions of the molecule. The constant domains of human Ig molecules can bind to the surface of macrophages via Fc receptors (FcR) to promote and enhance phagocytosis. These FcR are expressed on leucocytes and platelets but not on erythrocytes. Only IgG and IgM have the ability to interact with and initiate the complement cascade for the elimination of pathogens and the modulation of inflammation. IgG is the most important class of immunoglobulin in secondary immune responses, it is the only class able to cross the placenta and confer a high degree of passive immunity to the newborn. IgM is the predominant antibody in primary immune responses. IgD is a B cell receptor, IgA is primarily involved with the humoral protection of mucosal surfaces and IgE is thought to have evolved as an anti-parasite response but is now most commonly associated with allergic diseases such as asthma and hay fever (reviewed by Roitt (Roitt *et al.*, 2001)).

## **1.5 Antibody diversity**

### **1.5.1 Clonal theory of selection**

The clonal theory of selection is attributed to Burnet (Burnet, 1959) and provides an explanation of the specificity of the adaptive humoral immune response. Briefly, the theory states that an antigen selects a lymphocyte, from a variety of lymphocytes, with a receptor capable of reacting with part of that antigen. The selected lymphocyte is then activated to proliferate (clonal expansion) and secrete the cognate antibody. The concept of clonal selection proposed by Burnet in 1959 is in essence correct but we

now know that the selection and diversification of the humoral response is more complex.

### **1.5.2 Generation of antibody diversity**

There are four main ways in which to generate antibody diversity, three are consequences of the process of recombination of immunoglobulin genes used to create novel gene segments encoding complete immunoglobulin variable domains. Ig can either be membrane bound or secreted and this is regulated by alternative splicing. The carboxy-termini of the transmembrane and secreted forms of the Ig H chains are encoded in separate exons. The last exon of the constant region gene contains the sequence encoding the transmembrane region of the heavy chain. If the primary transcript includes all exons, sIg is produced. If transcription is terminated before the last exon, only the secreted molecule can be produced. The fourth is a mutational process that only occurs on the rearranged DNA encoding the variable domains. The sequence of the rearranged V genes can be altered by a process of somatic hypermutation (SHM), which can result in affinity maturation (Neuberger & Milstein, 1995). B cells in which hypermutation leads to an increased affinity for antigen will be preferentially selected and expanded. The site of mutations are not random but occur in hotspots (Rogozin *et al.*, 2001) corresponding to the CDR. The process of affinity maturation is coupled with class switching, which is an ordered process leading to the replacement of the original constant domain gene of the heavy chain ( $\mu$  or  $\delta$ ) by other constant domain genes ( $\gamma$ ,  $\epsilon$  or  $\alpha$ ) leading to the production of IgG, IgE or IgA.

### 1.5.3 Immunoglobulin gene recombination

The primary antibody repertoire is generated independent of antigen by a process of random gene rearrangement, and it is this rearrangement of Ig V domain genes, which is responsible for the generation of diversity of the amino acid sequence of the antigen-binding site. Each B cell expresses a sIg with a unique antigen-binding site. These are produced *de novo* in infinite permutations by the random recombination of each of two or three genes; variable (V), diversity (D) (Ig heavy chain only), and joining (J) gene (Figure 1.5), producing a diverse repertoire of up to  $10^{12}$  antigen binding specificities (Tonegawa, 1983). The position at which V and J (light chain) and D and J (heavy chain) join also varies. These slight differences in the position at which recombination occurs generates variations in the amino acid sequence leading to additional diversity. Furthermore, a few nucleotides may be inserted or deleted between D and JH and VH and D by the enzyme terminal deoxynucleotidyl transferase (TdT), which is referred to as N-region diversity.

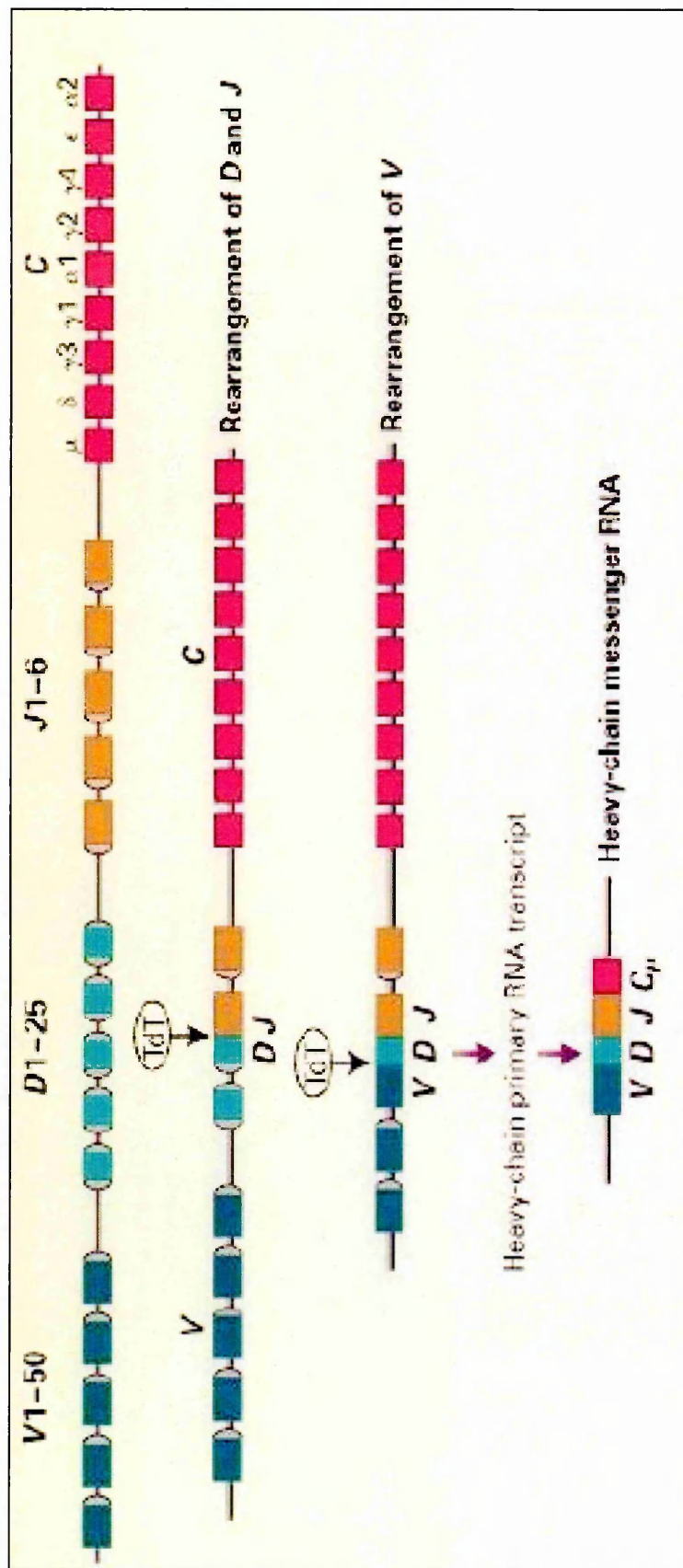


Figure 1.5 Immunoglobulin gene rearrangement on the heavy chain. Ig H domain locus on chromosome 14, band q32.33. Diversity (D) and Joining (J) genes rearrange first. The rearrangement of the Variable (V) gene occurs next and combines with the rearranged DJ. Finally the VDJ rearrangement combines with a Constant (C) gene to produce the messenger RNA. Reproduced with kind permission W H Ouwehand.



#### **1.5.4 Activation-induced cytidine deaminase**

Activation-induced cytidine deaminase (AID) is an essential enzyme in the regulation of class switch recombination (CSR), SHM (Muramatsu *et al.*, 2000) and gene conversion (GC) (Arakawa *et al.*, 2002) (reviewed by Honjo *et al.* (Honjo *et al.*, 2004)). Different species employ different strategies of diversification, eg mice and humans use exclusively hypermutation (Milstein & Rada, 1995), whereas rabbits, cows and pigs use mainly gene conversion (Butler, 1998). The gene for AID is induced by B cell stimulation and is homologous to the cytidine deaminase apolipoprotein B mRNA editing enzyme (APOBEC-1) gene (Muramatsu *et al.*, 1999), leading to the hypothesis that AID is a mRNA editing enzyme (Muramatsu *et al.*, 1999). However, further studies on the mechanism of SHM indicate that AID directly modifies DNA by deamination of cytosine (C) to uracil (U) (Di Noia & Neuberger, 2002).

AID expression closely correlates with the immunopathological phenotype of B-cell malignancies, therefore AID, as a genome mutator, may play a role in ongoing transformation of the germinal centre derived B cell non-Hodgkin's lymphomas (B-NHL) (Smit *et al.*, 2003).

##### **1.5.4.1 Somatic hypermutation**

The theory of SHM was one of the first hypotheses to explain the genetic-diversity of antibody-forming cells (Lederberg, 1959). SHM is the alteration of germline immunoglobulin sequences by introduction of nucleotide changes during the lifetime of a B-cell (Lederberg, 1959). Studies on Bence-Jones proteins established that



antibody diversity is largely confined to the amino-terminal (variable) domains of the polypeptide chains (Hilschmann & Craig, 1965; Milstein, 1966; Titani *et al.*, 1966). More recently the demonstration that SHM contributes substantially to the diversity of the antibody repertoire (Parham, 1998) and the discovery of several low-fidelity DNA polymerases encoded by the mammalian genome (Ohmori *et al.*, 2001) led to the understanding that the substantial proportions of the mutations generated by SHM are as a result of direct modification of the DNA duplex itself rather than from errors in copying the DNA (Neuberger *et al.*, 2005).

SHM can now be divided into two phases; AID dependent first phase is triggered by specific enzyme catalysed deamination of C (deoxycytidine) residues to U (deoxyuracil) residues. Resulting in the generation of a lesion composed of U mispaired with G. Replication over the U.G mispair could be responsible for generating many (or possibly all) of the mutations at G.C pairs. The second phase of SHM depends on the recognition of the DNA lesions generated in the first phase, leading to mutations that are mainly (or possibly all) at A.T (deoxyadenosine.deoxythymidine) pairs, but the mechanism for this is still unknown (Neuberger *et al.*, 2005). Recombinant AID has the predicted DNA-deaminating activity, and its preference for C residues with adjacent 5' -(A/T)-(A/G) sequences (Petersen-Mahrt *et al.*, 2002; Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Pham *et al.*, 2003) supports the sequence of intrinsic mutation hot spots in Ig genes (Rada *et al.*, 1998; Rogozin *et al.*, 2001).

#### 1.5.4.2 Class switch recombination

CSR is a region-specific recombination that switches isotypes from IgM or IgD to IgG, IgA or IgE contributing diverse effector functions to immunoglobulin with a given antigen specificity. The gene for the heavy chain is cut on the 3' side of the assembled V-region segments and the assembly moved to the 5' side of another of the CH gene segments, upstream in the locus. The ability of a B cell to switch CH gene segments depends on its receiving help from helper T cells.

AID-deficient mice show the complete loss of class switching and accumulation of IgM in sera and faeces (Muramatsu *et al.*, 2000). Patients with the autosomal recessive disease, hyper IgM syndrome type-2 (HIGM2) have severe defects in class switching (Revy *et al.*, 2000). Genetic linkage analysis has revealed that the mutation is mapped to chromosome 12p13, which coincides with the AID gene locus (Muto *et al.*, 2000). All HIGM2 patients have mutations in the AID gene and are defective in CSR (Ta *et al.*, 2003).

#### 1.5.4.3 Gene conversion

GC modifies an antibody by swapping part of its antigen-binding region for a replacement gene segment. Rabbits, cows and pigs favour gene conversion. Chicken B cells first develop their Ig repertoire by gene conversion in the bursa (Reynaud *et al.*, 1987; Arakawa & Buerstedde, 2004) and later fine-tune it by hypermutation in splenic germinal centres (Arakawa *et al.*, 1996).

AID is a requirement for all three gene diversification processes. However, the cytosine deamination activity must be further regulated, because only differences in the type, the location or the processing of the AID-induced DNA modification can explain the selective occurrence of recombination or hypermutation in different species and B cell environments. Based on the finding that certain AID mutations affect CSR but not SHM, it was suggested that AID needs the binding of a cofactor to initiate CSR (Barreto *et al.*, 2003; Ta *et al.*, 2003; Basu *et al.*, 2005).

### **1.5.5 Receptor editing**

Receptor editing is a phenomenon that occurs in the bone marrow and serves to rescue immature self-reactive B cells. The clonal selection theory anticipated that a random collection of Igs would include self-reactive specificities that require silencing (Burnet, 1959). The tolerization of these autoreactive B cells was deduced using transgenic mice carrying antibody genes coding for self-reactive Igs (Goodnow *et al.*, 1988; Nemazee & Burki, 1989; Gay *et al.*, 1993; Tiegs *et al.*, 1993; Tze *et al.*, 2000). It was shown that the autoreactive B cells confronted with antigen were eliminated, anergised or the Ig specificity altered by continued gene recombination (receptor editing). It has been shown in Igk knock-in mouse strains that 25% of the light chains found on the surface of developing B cells in vivo are produced by receptor editing (Casellas *et al.*, 2001), however, it has not been confirmed whether all these replacements are induced by self-reactivity. This data is strongly suggestive that receptor editing plays an important role in the generation of the normal antibody repertoire.

## 1.6 Lymphoma

Lymphoma is a general term for a malignancy of lymphocytes. The prefix "lymph-" indicates the origin of the malignancy and the suffix "-oma" is derived from the Greek word meaning "tumour". Lymphomas result from a chromosomal translocation of a lymphocyte with subsequent further acquired DNA mutations resulting in a malignant transformation. These acquired genetic events cause uncontrolled and excessive clonal cell proliferation conferring a survival advantage on the malignant lymphocyte and its progeny. The accumulation of these dividing cells results in the tumour masses in the lymph nodes and other sites. Traditionally lymphomas can be divided into two categories: Hodgkin lymphoma or Hodgkin's disease (HD) named after Thomas Hodgkin, an English physician who described several cases in 1832, and non-Hodgkin lymphomas (NHL), a term covering all other lymphomas. HD is histologically distinguished from NHL by the presence of large bilobed nuclear cells known as Reed-Sternberg (RS) cells (Sternberg, 1898; Reed, 1902) and represents ~13% of all the lymphomas diagnosed annually.

### 1.6.1 Non-Hodgkin Lymphoma

The Revised European-American Lymphoma (REAL) classification (Harris *et al.*, 1994) lists more than 20 types of NHL divided between B- and T-cell lymphomas. B-cell lymphomas are a diverse group of diseases that are classified histologically into low, intermediate and high-grade varieties. B-cell lymphomas account for about 87% of all cases of NHL and approximately 40% of these are of the low-grade variety.

### **1.6.2 Follicular Lymphoma**

Follicular lymphoma (FL) is the most common type of slow-growing or indolent non-Hodgkin's lymphoma. FL is named because the malignant cells usually grow in a circular or follicular pattern in the lymph nodes. FL occurs in the germinal centre B cell after the mature B cell has encountered antigen (Figure 1.1).

### **1.6.3 Development of Follicular Lymphoma**

FL originates from B-cell lymphocytes developing and maturing in the lymphoid tissues. FL tumours are characterised by the presence of small-cleaved cells (centrocytes) or large cells (centroblasts) in the lymphatic system (Figure 1.6).

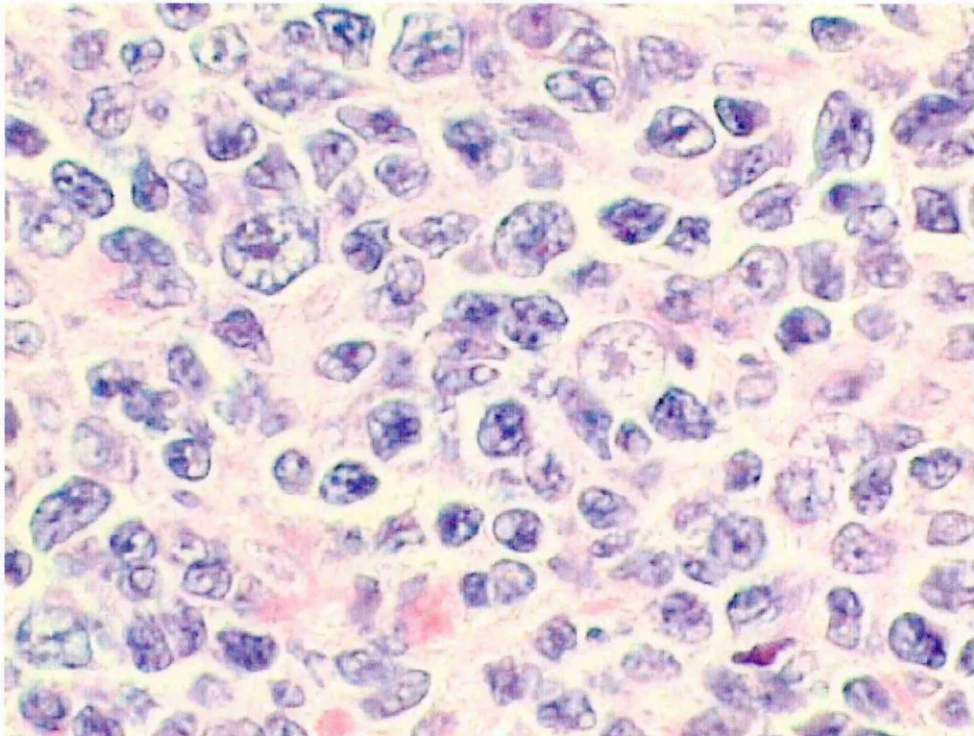


Figure 1.6 Lymph node section from patient with follicular lymphoma. Paraffin mounted section of lymph node stained with Haematoxylin and Eosin. High power magnification showing a mixture of centrocytes and centroblasts. Centrocytes are the small cleaved cells and the centroblasts are the large non-cleaved cells. Reproduced from [http://pleiad.umdj.edu/~dweiss/follicular/grade1\\_fcc\\_img.html](http://pleiad.umdj.edu/~dweiss/follicular/grade1_fcc_img.html).

Typically, the malignant masses are dominated by centrocytes, even though centroblasts are always present. In rare cases, a patient may have a FL that consists almost entirely of centroblasts.

The lymphoma can transform and become more aggressive over many years, and the follicular pattern may change from a lower percentage to primarily centroblasts.

Although the lymphoma usually begins in lymphoid tissue, it is not uncommon for metastases to form in the bone marrow and peripheral blood.

The chromosomal translocation t(14:18)(q32;q21) is present in over 80% of NHL (Hoffbrand & Pettit, 1994). The translocation involves the immunoglobulin heavy chain locus (JH Ig H) on chromosome 14 and the bcl-2 gene on chromosome 18. Bcl-2 is an anti-apoptotic protein that resides in the outer mitochondrial membrane (OMM) and the membrane of the endoplasmic reticulum (ER). Over expression of bcl-2 is known to block the release of cytochrome c into the cytoplasm thereby preventing activation of caspase-9 and -3 preventing caspase induced apoptosis (Zamzami *et al.*, 1998). The bcl-2 IgH fusion gene in FL is deregulated, leading to the transcription of excessively high levels of the anti-apoptotic bcl-2 protein (Vaux *et al.*, 1988), thus decreasing the propensity of these cells to undergo apoptosis (Yunis *et al.*, 1987). This confers a survival advantage and therefore a gradual accumulation of the malignant cells.

#### **1.6.4 Incidence of Follicular Lymphoma**

FL is the second most common lymphoma in the United States and Western Europe. About one in five people with non-Hodgkin's lymphoma have FL, and in more than two thirds of patients the low-grade lymphoma is of the follicular type (Glass *et al.*,

1997). FL usually affects middle-aged and elderly people. It is rarely diagnosed in children or adolescents. The median age at the time of diagnosis is 60 years; it is slightly more frequent in women than men and is more common in white Caucasoid than in Asian or black populations. In the UK the recorded incidence is currently 4/100,000 and is increasing at about 5% a year.

### **1.6.5 Classification**

The World Health Organization (WHO) classifies FL into three grades, low, intermediate and high. These are based on the number of centroblasts in the tumour sections under high field microscopy. In short: Grade I, a patient has fewer than five centroblasts, this is the most common type. Grade II, a patient has between six and fifteen centroblasts. Grades I and II are considered to be indolent or slow growing. Grade III, a patient has more than 15 centroblasts; this is a rare more aggressive type.

### **1.6.6 Signs and symptoms**

In most cases, patients seek medical advice because of the appearance of swollen glands in the neck, armpits or groin. These swollen lymph nodes are mostly painless and can be present for several weeks before attention is directed toward them. General symptoms may include feeling tired, having a flu-like syndrome or aching all over. Others experience night sweats and some may have recurring high-grade or constant low-grade fevers. Since all these symptoms are common to many illnesses, from minor ailments to serious disorders, the correct diagnostic procedures must be performed in order to confirm or rule out the presence of lymphoma.



### **1.6.7 Diagnosis**

At the time of diagnosis the vast majority of patients tend to have widespread, advanced-stage disease. However, as FL is slow growing and indolent that will progress slowly, without any form of treatment, often showing a tendency to wax and wane with frequent spontaneous regressions (23%) and complete remissions (8%)(Horning & Rosenberg, 1984).

A diagnosis of FL is confirmed by microscopic examination of a lymph node biopsy. Blood tests, X-rays, computed tomography (CT) and magnetic resonance imaging (MRI) scans, and bone marrow samples are also taken to obtain more information about the type of lymphoma and the extent to which the disease has spread (staging). FL has 4 stages of disease progression, stage I - one group of lymph nodes (LN) is affected. Stage II – more than two groups are affected. These groups are either above or below the diaphragm. Stage III - more than two groups are affected with locations both above and below the diaphragm. The spleen may also be affected. Stage IV – The lymphoma has spread beyond the LN to other organs ie, bone marrow, peripheral blood, liver and lungs. The stage of the lymphoma is crucial in deciding the most effective course of treatment. It is important to note that at first presentation 40% of patients have spleen involvement and 60-70% have bone marrow involvement.

### **1.6.8 Disease progression, treatment and prognosis**

Disease progression varies from patient to patient depending on the speed of tumour growth and the involvement of organs other than LN. Disease acceleration is often

associated with progression to a more aggressive histological pattern and a worsening prognosis.

FL responds well to conventional therapies including radiotherapy, single-agent chemotherapy, aggressive combination chemotherapy and more recently, combination therapies including the monoclonal anti-CD20 (Rituximab) (Maloney *et al.*, 1997a; Maloney *et al.*, 1997b). Very high dose chemotherapy followed by autologous bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) is performed on patients with recurrent or refractory low-grade NHL. However, patients will inevitably relapse and after each relapse the trend is for shorter remissions until the disease becomes refractory to treatment. Allogeneic BMT has been shown to induce longer remissions, but transplant-associated mortality is 10-15% higher than with autologous BMT (Verdonck *et al.*, 1997). Sometimes patients may have no symptoms for many years and as there is no evidence that early medical intervention can cure the disease or prolong survival a close observation "watch and wait" policy is generally adopted. For others, treatment may be required for alleviation of symptoms, such as fever, night sweats, weight loss, pain, obstructions or the development of anaemia or other cytopenias. However, first treatment usually results in complete or partial remission.

Overall the prognosis for FL patients is not good, the median survival is approximately 10 years regardless of the therapeutic modalities employed or when treatment was commenced.

## **1.7 DNA vaccines**

Vaccine development has come a long way since Edward Jenner's experiments in the

late 18<sup>th</sup> century (Jenner, 1798). The serendipitous discovery that intramuscular injection of naked plasmid DNA could express protein from a gene cloned under a relevant promoter prompted a new era in vaccine technology. The first demonstration of this performed by Wolff and colleagues who coincidentally showed expression of cloned genes for chloramphenicol acetyltransferase, luciferase and  $\beta$ -galactosidase (Wolff *et al.*, 1990) after intramuscular delivery of plasmid DNA (which was used as a negative control construct). Expression was shown to be localised to the muscle and persisted for over 19 months (Wolff *et al.*, 1992). Immune responses against the products of such genes were demonstrated with DNA plasmids encoding luciferase (Tang *et al.*, 1992) and HIV gp120 (Felgner & Rhodes, 1991). The potential of DNA vaccination was realised when it was shown that protection from influenza A could be established in mice immunised with plasmid DNA encoding the nucleoprotein (NP) of influenza A (Ulmer *et al.*, 1993; Yankauckas *et al.*, 1993). Furthermore, it was demonstrated that DNA vaccination led to the generation of long-lived humoral and/or cellular immune responses against a range of antigens (Donnelly *et al.*, 1994; Donnelly *et al.*, 1997).

Typically, DNA vaccines are bacterial plasmids carrying genes encoding pathogen or tumour antigens. Most vaccine plasmids share the same four basic features. Firstly, an origin of replication (ori) suitable for producing high yields of plasmid in *E. coli*. Secondly, an antibiotic resistance gene for selection of *E. coli* bearing plasmid. Thirdly, a strong eukaryotic enhancer/promoter for high-level expression of the antigen gene. Finally, mRNA transcript termination/polyadenylation sequence to direct protein expression in mammalian cells.

These four elements are the basic requirement for replication of and expression from plasmid DNA. The ColE1 plasmid derived bacterial origin of replication is known to

provide high yields of plasmid DNA during bacterial growth, and antibiotic selection is required so that only *E. coli* transformed with the plasmid replicate. Several high level expression promoters have been used in DNA vaccination protocols, including human cytomegalovirus immediate/early promoter (pCMV) (Boshart *et al.*, 1985; Furth *et al.*, 1991), Rous sarcoma virus LTR (pRSV) (Gorman *et al.*, 1982), and Simian virus 40 early promoter (pSV40) (Wenger *et al.*, 1994). These have been used in conjunction with the SV40 or bovine growth hormone 3'-untranslated region transcript termination/polyadenylation sequences (Pfarr *et al.*, 1986). Although promoter use differs between studies all those mentioned above give high level expression *in vivo* and comparison of promoters shows no clear differences between them (Wenger *et al.*, 1994; Barnhart *et al.*, 1998). Expression of mammalian genes also benefits from the inclusion of an intron between the promoter and methionine (AUG) start codon (Chapman *et al.*, 1991). Other regulatory elements include a Kozak sequence 5' of the AUG codon which introduces modest secondary structure (hairpin loop approximately 14 nucleotides upstream of AUG) and improves recognition of the start codon (Kozak, 1990). A signal peptide sequence may also be included to direct protein expression through the Golgi and endoplasmic reticulum leading to secretion. Using these elements the cloned genes are transcribed and translated by somatic cells that have either been directly transfected with or taken up plasmid DNA. Initially it was thought that the vaccine plasmid might integrate into the genome of a transfected cell or induce anti-DNA autoantibodies similar to those produced in the autoimmune disease, systemic lupus erythematosus (SLE). These issues posed serious safety concerns for the use of DNA vaccines in humans. Low affinity DNA autoantibodies are present ubiquitously in the human population but induction of pathological anti-dsDNA autoantibodies is difficult. In normal mice DNA must be denatured,

complexed with methylated bovine serum albumin and co-administered with complete Freund's adjuvant in order to induce high affinity dsDNA autoantibodies (Gilkeson *et al.*, 1991). Predications of the frequency of integration/insertion events leading to tumorigenesis are below background levels (Kurth, 1995) and no integration of either part or all of plasmid DNA from vaccines has been found, even after persistence of episomal plasmid for long periods (Nichols *et al.*, 1995).

### 1.7.1 DNA vaccines in animal models

DNA vaccination has been used to raise immune responses to a number of antigens in a variety of species (eg chickens, mice, ferrets, cattle and non-human primates). Viral antigens include influenza virus haemagglutinin, matrix protein and nucleoprotein (Fynan *et al.*, 1993; Ulmer *et al.*, 1993), human immunodeficiency virus 1 (HIV-1) gp120, gp160 (Shiver *et al.*, 1995), env, gag and pol genes (Boyer *et al.*, 1997), bovine herpes virus gIV (Cox *et al.*, 1993), rabies virus surface glycoprotein (Xiang *et al.*, 1994), hepatitis B virus (HBV) (Davis *et al.*, 1985) surface antigen and hepatitis C virus (HCV) (Major *et al.*, 1995). Bacterial antigens include *Mycobacterium tuberculosis* heat shock protein 65 (Hsp65) and antigen 85 (Ag-85) (Huygen *et al.*, 1996; Tascon *et al.*, 1996) and tetanus toxin Fragment C (Anderson *et al.*, 1996). Parasitic antigens include malaria circumsporozoite protein (Doolan *et al.*, 1996; Leitner *et al.*, 1997), *Schistosoma japonicum* (Yang *et al.*, 1995) and *Leishmania major* gp63 (Xu & Liew, 1995).

### 1.7.2 Vaccination as cancer immunotherapy

Unlike most vaccines for infectious agents, cancer vaccination is therapeutic, involving attempts to activate immune responses against antigens of the tumour to which the immune system has already been exposed and tolerance may have been developed. Tumours are essentially self but over expression of self or modified self-protein, eg novel fusion proteins encoded by a fusion gene antigen, may provide a target which can be exploited in tumour vaccine design. To the immune system these antigens are very close to self. For cancer immunotherapy to be effective firstly the tumour mass must be reduced by classic chemo/radiotherapy or modern drugs eg Glivec (tyrosine kinase inhibitor for the treatment of chronic myeloid leukemia (CML)) or Rituximab (monoclonal anti-CD20 for the treatment of B-cell lymphomas). The power of the immune system can then be harnessed as a means to control minimal residual disease (MRD). One of the major challenges of cancer immunotherapy has remained the identification of suitable targets unique to the cancer cell, but with adequate antigenicity to elicit a clinically relevant immune response. The tumour target and its expression in tissues other than the tumour is therefore of primary importance for immunotherapy, together with an efficient method of delivering the antigen. Over the last two decades with the steep increase in the knowledge about the biology of malignant cells the list of candidate tumour antigens has increased exponentially. Serum antibodies in patients have also been used to identify potential tumour antigens by serological identification of antigens by recombinant expression (SEREX) profiling (Preuss *et al.*, 2002). With the completion of the human genome

project (Lander *et al.*, 2001) sequencing and gene-expression profiling of malignant cell populations has provided additional targets.

Studies using autologous tumours, such as the murine CT26 colon carcinoma model in Balb/c mice (Fearon & Vogelstein, 1990) or the C57BL/6 derived murine B16-F10 melanoma (Pardoll, 1995) have been used to induce tumour specific immune responses in mice. At around the same time DNA vaccination began to incorporate cytokines into vaccine vectors to improve antigen specific immunity, groups working in cancer immunotherapy began to use cytokines to enhance the immunogenicity of autologous tumours *in vivo*. Up until this point cancer vaccines consisted of killed tumour cells or lysates administered with Bacillus Calmette-Guerin (BCG) or similar adjuvants (Berd *et al.*, 1990). Tumours transduced with vectors expressing different cytokines produced a distinct profile of inflammatory reaction and immune cell involvement. For example, administration of IL-2 expressing tumours produces an inflammatory infiltrate of mainly CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes whilst IL-4 expressing tumours produce an inflammatory reaction consisting mainly of macrophages and eosinophils (Pardoll, 1995). Experimental work in human transfer experiments has shown that the A20 B cell lymphoma engineered to express costimulatory molecules CD80, CD86 and CD137L (4-1BBL), a TNF family protein expressed on activated T cells (Goodwin *et al.*, 1993), can confer long lasting tumour immunity against challenge with parental tumour (Guinn *et al.*, 1999).

### 1.7.2.1 DNA vaccines in cancer immunotherapy

Carcinoembryonic antigen (CEA), a membrane glycoprotein, is normally expressed only by human colorectal, gastric, pancreatic, breast, and non-small cell lung carcinomas (Kantor *et al.*, 1992), making it an excellent target for immunotherapy. Studies using DNA vaccines encoding CEA, in a CEA-transgenic mouse model have been used to prevent Lewis lung carcinoma (Niethammer *et al.*, 2001) and co-delivered with trimeric CD154 (CD40L) to protect against murine colon carcinoma (Xiang *et al.*, 2001). Co-delivery of CEA with Hepatitis B surface antigen (HBsAg) in a human clinical trial with metastatic colorectal carcinoma patients showed lymphoproliferative responses to CEA (Conry *et al.*, 2002). Recombinant CEA expressing vaccinia virus has also been used to induce cell mediated immunity and specific antibodies, which protect (in the murine model) from challenge with murine colon adenocarcinoma expressing human CEA (Kantor *et al.*, 1992).

By far some of the most successful antigen targets used in immunotherapy are those found in melanoma. Of the 14 cancer vaccines in phase 3 clinical trials (Reichert & Paquette, 2002) 6 are against melanoma, one of which is a DNA vaccine (Allovectin-7, VICAL). The melanoma antigens are attractive targets because their expression is restricted to skin melanocytes. Those used for vaccination are melanocyte differentiation antigens MART-1, MAGE, MelanA, tyrosinase, tyrosinase-related peptide –1, and gp100. However vaccination with these antigens also illustrates the paradigm of cancer immunotherapy, since they also induce autoimmune vitiligo (Rosenberg & White, 1996; Steitz *et al.*, 2002) due to the anti-tumour response also targeting normal healthy melanocytes.



### 1.7.2.2 Vaccines as therapy for lymphoma

Another immunogenic tumour specific target is idiotype (Slater *et al.*, 1955; Lynch *et al.*, 1972). In B cell lymphomas and myelomas, the idiotypic determinants of the Ig are clonal and unique, thus serving as a marker for the malignant B cell clone (Stevenson & Stevenson, 1975). It has long been established that anti-idiotype responses can be induced after vaccination with idiotype, first described in animals immunised with myeloma proteins (Lynch *et al.*, 1972). This led to immunisation of mice with purified protein from the tumour itself (Stevenson & Gordon, 1983), an approach which is still being assessed experimentally for lymphoma and multiple myeloma using IL-2 and GM-CSF as adjuvant (Stritzke *et al.*, 2003). Later, vaccination with idiotype in the form of the 38C13 murine lymphoma conjugated to an immunogenic carrier such as keyhole limpet haemocyanin (KLH) was shown to protect from tumour challenge in the murine model (George *et al.*, 1987; Kaminski *et al.*, 1987; George *et al.*, 1988). In patients with B cell lymphoma Ig protein prepared from the patients tumour by heterohybridoma fusion was used to induce anti-idiotypic responses. Idiotype, either alone (Hsu *et al.*, 1997), chemically linked to KLH (Kwak *et al.*, 1992) or administered with recombinant GM-CSF (Bendandi *et al.*, 1999) have been evaluated in patients with FL. All strategies induced anti-idiotype with the latter inducing molecular remission in 8/11 patients (Bendandi *et al.*, 1999). Idiotype conjugated to KLH or idiotype alone have also been used to pulse DCs, which were subsequently used as a vaccine again using the 38C13 model in mice (Hsu *et al.*, 1996; Timmerman *et al.*, 2001). These approaches demonstrated some limited success, however the idiotype target for each patient is unique and therefore requires the laborious and time consuming production of patient specific idiotype protein.

The advent of DNA vaccines made the production of patient specific idiotype vaccines more feasible. DNA vaccines can be constructed to express patient specific idiotype relatively easily and as described can readily incorporate adjuvants to boost immunogenicity. Fusion with fragment C (FrC) of tetanus toxoid has produced effective protection from challenge against the A31 B cell splenic lymphoma and 5T33 *in vitro* myeloma cell line, in mice (King *et al.*, 1998), demonstrating the effectiveness of using both idiotype as target, and FrC as an adjuvant to provide T cell help in DNA vaccination. The mechanism of tumour protection after DNA vaccination with idiotype has been shown to be antibody mediated and is largely accepted to be via antibody-dependant cell cytotoxicity (ADCC) (Syrenelas & Levy, 1999). More recently effective anti-tumour responses have been achieved following immunisation of mice with an idiotype bearing A20 tumour transduced with adenoviruses encoding CD154 (Briones *et al.*, 2002) and with adenovirus encoding idiotype fused to mouse kappa constant domains (Timmerman *et al.*, 2001).

### **1.7.3 Clinical trials of DNA vaccines in FL**

The first clinical trial using DNA vaccines encoding the patients idiotype as a treatment for FL was performed by the Cambridge and Southampton teams in 1997 (Hawkins *et al.*, 1997). The primary objective of the phase I trial CRC no. 92/33, protocol number PH1/027 was to establish the safety of the intramuscular delivery of plasmid DNA in idiotypic vaccination. The follow up to this trial was a phase I/II trial CRC no. 98/04, protocol number PH2/039, using the DNA plasmid, encoding idiotype fused to fragment C of tetanus toxoid (pVAC2) (King *et al.*, 1998). The trial was a dose escalation study designed to evaluate the clinical efficacy of the plasmid pVAC2

in eliciting an anti-id response in FL patients in first remission. The trial differed from phase I in two ways. Firstly, the plasmid pVAC2 versus the adjuvant free pVAC1 and secondly the disease state, first remission versus end stage. The preparation of the vaccines, their evaluation in the rabbit model and the assessment of the anti-idiotypic and anti-tetanus response in FL patients are presented in chapters 3 and 4 of this thesis. A similar approach was attempted in FL patients by immunising with a DNA vaccine encoding patient derived VH/VL domains and mouse constant domains (Timmerman *et al.*, 2002). The majority of patients displayed an immune response against the mouse immunoglobulin, however specific responses against idiotype were low, with only one patient displaying T-cell proliferation against idiotype.

As with infectious diseases, clinical trials of idiotype based DNA vaccines have been clinically less effective as in the murine models of B cell lymphoma. It is of interest to note that despite the extensive research in DNA vaccination not many of the clinical trials have made it into phase III. In fact, during the phase I/II clinical trial described in this thesis evidence that DNA vaccination alone was not sufficient to induce the necessary immune responses was reported (Hanke *et al.*, 1998). The next phase of genetic vaccines is well underway combining a DNA vaccine prime immunisation, followed by a powerful boost immunisation delivered by a recombinant viral vector, eg modified vaccinia ankara (MVA) (prime-boost). Phase I/II clinical trials for malaria (McConkey *et al.*, 2003; Moorthy *et al.*, 2003) and HIV (Mwau *et al.*, 2004; Cebere *et al.*, 2005) have produced promising results. However, the first generation T cell-inducing DNA/MVA against a multiple epitope *Plasmodium falciparum* vaccine was ineffective at reducing the natural infection rate in semi-immune African adults (Moorthy *et al.*, 2004a; Moorthy *et al.*, 2004b). A prime-boost vaccine has been

developed against tuberculosis (TB) and is currently entering into a phase III trial (personal communication with Prof Adrian Hill).

Despite the promise of prime-boost as an effective vaccination strategy it would not be a suitable approach as a therapeutic for FL. Each idiotypic vaccine is patient specific and the time required to produce individual MVA makes it unviable.

## 1.8 Aims

A phase III clinical trial using DNA vaccination as a treatment for Follicular Lymphoma provides the central theme for my research. In the following chapters I will describe the modification of an Invitrogen plasmid to produce Calmodulin tagged recombinant scFv in *Drosophila* S2 cells, the production and sterility and quality testing of clinical grade DNA vaccines and the subsequent antibody responses generated in Dutch rabbits and patients post vaccination.

The final chapter, describes a single case report, bringing together all the techniques previously described in order to provide a set of reagents to molecularly support the hypothesis that a patients' low-grade FL was responsible for their life threatening thrombocytopenia.

## **Chapter 2 – Materials and Methods**

### **2.1 Materials**

#### **2.1.1 List of Equipment**

The following laboratory equipment were routinely used for this work

AIC Alpha Imager 2000 gel documentation system

ALC PK131 and 131R bench-top centrifuges

Applied Biosystems 3100 Genetic Analyser system

Beckman Avanti J-25I centrifuge

Biometra Trio thermoblock

Biorad Miniprotean III (PAGE gels)

Cambridge Electrophoresis EM100 minigel tank (DNA gels)

Dynex MRX 5000 microtitre plate reader

Equibio Easyject plus electroporator

FT Applikon ADI 1030 Bioconsole Fermentor

Grant controlled temperature water bath

Kikusui PMC350-0.2A power supply

Millipore Miliblot System

MSE Micro Centaur microcentrifuge

New Brunswick controlled temperature shaker/incubator

P-1 peristaltic pump, Amersham Biosciences  
PROline microchef ST44 microwave  
Shimadzu UV mini 1240 UV-Vis spectrophotometer  
Techne DB-2A Dri-block

### 2.1.2 Reagents

Analytical grade reagents were supplied primarily by either Sigma (Poole, UK) or BDH (Poole, UK). Details of suppliers of other reagents are listed below

|                               |                   |
|-------------------------------|-------------------|
| Applied Biosystems (ABI)      | Warrington, UK    |
| Aldevron                      | ND, USA           |
| Alexis Biochemicals           | Nottingham, UK    |
| Ambion                        | Huntingdon, UK    |
| Amersham Pharmacia            | Bucks, UK         |
| Beckman Coulter               | High Wycombe, UK  |
| Biorad                        | CA, USA           |
| Becton Dickinson (Pharmingen) | Oxford, UK        |
| Boehringer Mannheim           | Mannheim, Germany |
| Corning                       | High Wycombe, UK  |
| HT Biotechnology              | Cambridge, UK     |
| Invitrogen                    | Grotingen, NL     |
| Jackson Immunoresearch        | Luton, UK         |
| JRH Biosciences               | KS, USA           |
| Gibco-BRL                     | Paisely, UK       |

|                              |                 |
|------------------------------|-----------------|
| Medicell international       | London, UK      |
| NBS Biologicals              | Huntingdon, UK  |
| New England Biolabs (NEB)    | Hitchin, UK     |
| National Blood service (NBS) | Bristol, UK     |
| NIBSC                        | Herts, UK       |
| Pierce                       | Cheshire, UK    |
| Promega                      | Southampton, UK |
| Qiagen                       | Dorking, UK     |
| R&D Systems                  | Abingdon, UK    |
| Roche                        | IN, USA         |
| Sigma-Genosys                | Cambridge, UK   |
| Stratagene                   | Cambridge, UK   |
| VWR International            | Poole, UK       |

### 2.1.2.1 pVAC2-scFv constructs

The patients for the clinical trial were enrolled by three centres. Royal Bournemouth Hospital, Bournemouth (B), Christie Hospital, Manchester (M) and Southampton General, Southampton (S).

| STUDY No. | Heavy Chain                          | Closest Germline | Light Chain   | Closest Germline   |
|-----------|--------------------------------------|------------------|---|--------------------|
| B01       | V <sub>H</sub> 4 / J <sub>H</sub> 4b | V4-59            | V <sub>λ</sub> 7 / J <sub>λ</sub> 2                   | 14-7               |
| B02       | V <sub>H</sub> 3 / J <sub>H</sub> 4a | P1               | V <sub>κ</sub> 4 / J <sub>κ</sub> 1                   | DPK 24             |
| B03       | V <sub>H</sub> 3 / J <sub>H</sub> 6b | V3-48            | V <sub>κ</sub> 1 / J <sub>κ</sub> 1                   | A30                |
| B04       | V <sub>H</sub> 3 / J <sub>H</sub> 3a | V3-48            | V <sub>κ</sub> 3 / J <sub>κ</sub> 1                   | DPK 22/A27         |
| B05       | V <sub>H</sub> 3 / J <sub>H</sub> 6b | V3-23            | V <sub>κ</sub> 1 / J <sub>κ</sub> 4                   | A30                |
| M01       | V <sub>H</sub> 4 / J <sub>H</sub> 5a | V4-39            | V <sub>κ</sub> 4 / J <sub>κ</sub> 1                   | DPK 24             |
| M02       | V <sub>H</sub> 4 / J <sub>H</sub> 4b | V4-39            | V <sub>κ</sub> 1 / J <sub>κ</sub> 5                   | DPK 8              |
| M03       | V <sub>H</sub> 3 / J <sub>H</sub> 6b | V3-23            | V <sub>λ</sub> 2 / J <sub>λ</sub> 2/3a                | DPL 11             |
| M04       | V <sub>H</sub> 1 / J <sub>H</sub> 4a | V1-02            | V <sub>κ</sub> 1 / J <sub>κ</sub> 3                   | DPK 1              |
| M05       | V <sub>H</sub> 1 / J <sub>H</sub> 4b | V1-02            | V <sub>λ</sub> 1 / J <sub>λ</sub> 2/3a                | DPL 8              |
| M06       | V <sub>H</sub> 3 / J <sub>H</sub> 5b | V3-23            | V <sub>κ</sub> 4 / J <sub>κ</sub> 4                   | DPK 24             |
| M08       | V <sub>H</sub> 3 / J <sub>H</sub> 4a | V3-15            | V <sub>λ</sub> 2 / J <sub>λ</sub> 2/J <sub>λ</sub> 3a | 2c.118D9/V1-2      |
| S01       | V <sub>H</sub> 4 / J <sub>H</sub> 2  | V4-34            | V <sub>κ</sub> 3 / J <sub>κ</sub> 2                   | DPK 22             |
| S02       | V <sub>H</sub> 3 / J <sub>H</sub> 4b | V3-21            | V <sub>λ</sub> 1 / J <sub>λ</sub> 2                   | DPL 2              |
| S03       | V <sub>H</sub> 3 / J <sub>H</sub> 6c | V3-11            | V <sub>κ</sub> 3 / J <sub>κ</sub> 4                   | V <sub>κ</sub> 3   |
| S04       | V <sub>H</sub> 3 / J <sub>H</sub> 4b | V3-21            | V <sub>κ</sub> 3 / J <sub>κ</sub> 4                   | V <sub>g</sub> 38K |
| S05       | V <sub>H</sub> 3 / J <sub>H</sub> 4a | V3-48            | V <sub>κ</sub> 1 / J <sub>κ</sub> 5                   | DPK 9              |
| S06       | V <sub>H</sub> 3 / J <sub>H</sub> 4b | V3-30            | V <sub>κ</sub> 1 / J <sub>κ</sub> 2                   | DPK 9              |
| S07       | V <sub>H</sub> 3 / J <sub>H</sub> 4b | V3-48            | V <sub>κ</sub> 1 / J <sub>κ</sub> 2                   | DPK 9              |
| S09       | V <sub>H</sub> 3 / J <sub>H</sub> 3  | V3-48            | V <sub>κ</sub> 1 / J <sub>κ</sub> 1                   | DPK 9              |
| S011      | V <sub>H</sub> 4 / J <sub>H</sub> 1  | V4-34            | V <sub>κ</sub> 4 / J <sub>κ</sub> 2                   | DPK 24             |
| S012      | V <sub>H</sub> 2 / J <sub>H</sub> 5b | V2-05            | V <sub>κ</sub> 1 / J <sub>κ</sub> 1                   | HK 102             |
| S013      | V <sub>H</sub> 3 / J <sub>H</sub> 4b | V3-48            | V <sub>κ</sub> 3 / J <sub>κ</sub> 1                   | DPK 22/A27         |
| S017      | V <sub>H</sub> 3 / J <sub>H</sub> 3b | V3-23            | V <sub>λ</sub> 2 / J <sub>λ</sub> 2                   | DPL 11             |
| S018      | V <sub>H</sub> 3 / J <sub>H</sub> 6b | V3-15            | V <sub>κ</sub> 1 / J <sub>κ</sub> 4                   | A30/SG3            |

Table 2.1 Closest germlines of patient scFvs. The closest germline sequences from the isolated patient V<sub>H</sub> and V<sub>L</sub> genes were identified. The scFvs were constructed and supplied in pVAC2 from the centres in Bournemouth, Southampton and Manchester.



### 2.1.3 Commonly used buffers

|  |   |
|--|---|
| PBS  | 125 mM NaCl, 25 mM NaH <sub>2</sub> PO <sub>4</sub> (pH 7.0).   |
| TBS  | 150 mM NaCl, 50 mM Tris-HCl (pH 7.4).   |
| TBE  | 0.09 M Tris, 0.09 M borate, 2 mM EDTA (pH 8.0).   |
| Borate coating buffer                            | 1 M Boric acid pH to 8.3 with NaOH.   |
| SDS PAGE buffer (5x stock)                       | 25 mM Trizma base, 250 mM glycine, 0.1% SDS.  |
| Transblot buffer                                 | 39 mM glycine, 48 mM Trizma Base, 0.037% SDS, 20% methanol.   |
| Coomassie blue stain                             | 0.25 g Coomassie brilliant blue, 10% glacial acetic acid, 10% iso-propanol in dH <sub>2</sub> O.              |
| Destain  | 20% methanol, 10% glacial acetic acid in dH <sub>2</sub> O.   |
| Immobilised Metal chelate                        | 0.25 M Na <sub>2</sub> HPO <sub>4</sub> , 0.25 M NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O,          |
| Affinity Chromatography (IMAC) buffer (5x stock) | 2.5 M NaCl, pH to 7.5 with orthophosphoric acid.  |
| DNA/RNA agarose gel loading bufffer              | 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.   |
| 2 x laemelli loading dye                         | 10% glycerol, 0.1% bromophenol blue, 2% SDS in 50 mM Tris. For reducing conditions add 10% β-mercaptoethanol. |

#### 2.1.4 Media for bacterial culture

Bacterial growth media 2xTY and all culture plates were routinely supplied by the Laboratory of Molecular Biology central facility. Bacteria transformed with antibiotic resistant plasmids were grown on plates and in culture with 100µg/ml.

#### 2.1.5 Bacterial Strains

Genotypes of the bacterial strains used this work are summarised below.

TOP10 : F' mcrAΔ(mrr-hsdRMS-mcrBC)Φ80lacZM15 ΔlacX74 deoR recA1

araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG

DH5α : F' Φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17

(r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>+</sup>) phoA supW44λ<sup>thi</sup>-1 gyrA96 relA1

#### 2.1.6 Plasmids

The DNA vaccination vector pVAC2 was constructed in the Department of Haematology, University of Cambridge by Dr Ian Harmer. Based on pcDNA3 (Invitrogen), pVAC2 (Appendix D3) contains the gene for fragment C (FrC) of tetanus toxoid (TT). The multiple cloning site (MCS) was designed to be compatible with the phage display plasmid pHenIX and the *E. Coli* expression plasmid pUC119-His used in this laboratory. Antigen was cloned as an SfiI / NotI fragment, the restriction sites SfiI, NcoI (5') and Not I (3') were routinely appended to the antigen genes by PCR.

## **2.2 Methods**

### **2.2.1 Molecular Biology**

#### **2.2.1.1 RNA Extraction**

For RNA extraction from a bone marrow biopsy approximately  $1 \times 10^7$  cells were homogenized in TRIzol (Invitrogen, NL). The homogenate was incubated at room temperature (RT) for 5 min. 0.2 ml of chloroform was added per 1 ml homogenate. The sample was shaken vigorously for 15 sec, and incubated at RT for 2-3 min. Subsequently the homogenate was centrifuged at 13000 g for 15 min at 4°C. The upper aqueous phase, containing the RNA, was transferred to a fresh tube and 0.5 ml of isopropanol was added. The samples were incubated at RT for 5-10 min before centrifugation at 13000 g for 10 min at 4°C. The pellet was washed by vortex resuspension in 1 ml of 75% ethanol. Subsequently the RNA was centrifuged at 7500 g for 5 min at 4°C. The pellet was air dried in a laminar flow hood before resuspension in diethyl-pyrocabonate (DEPC) treated water. To allow for complete resuspension the sample was heated to 55°C for 10 min before being quantified (Chpt 2.2.1.2). The integrity of the ribosomal RNA was checked by agarose gel electrophoresis (Chpt 2.2.1.3). For long-term storage the RNA was maintained at -70°C without significant degradation.

### 2.2.1.2 Nucleic Acid Quantification

RNA and DNA were quantified by spectrophotometry at wavelengths of 260 and 280 nm. An optical density (OD) of 1.0 corresponds to approximately 40  $\mu\text{g/ml}$  of RNA, 50  $\mu\text{g/ml}$  of double stranded DNA and 20  $\mu\text{g/ml}$  of single stranded DNA. Therefore the concentration of nucleic acids can be calculated with the following equations:

$$\text{RNA } \mu\text{g/ml} = \text{OD}_{260} \times 40 \times \text{dilution factor}$$

$$\text{dsDNA } \mu\text{g/ml} = \text{OD}_{260} \times 50 \times \text{dilution factor}$$

The 260/280 ratio for both RNA and DNA provides an estimate of the purity of the sample. A pure sample, without contaminants such as phenol or protein, gives a ratio between 1.7 and 2.0, where 2.0 is the most pure. At ratios lower than 1.6 accurate quantification of nucleic acid is not possible.

### 2.2.1.3 Agarose Gel Electrophoresis

Molecules of linear double stranded DNA migrate through gel matrices at rates that are inversely proportional to the  $\log_{10}$  of the number of base pairs (Helling *et al.*, 1974). The amount of agarose in the gel (% [w/v]) was determined based on the size of the DNA in base pairs; larger molecules need less agarose to separate efficiently. A 0.9% agarose gel has an efficient range of separation of linear DNA molecules of 0.5-7 kb. The appropriate amount of agarose (High pure low EEO agarose, Cambridge UK) was dissolved in 50 ml TBE (Chpt 2.1.3) by heating. The agarose was cooled and

2  $\mu$ l of the intercalating dye ethidium bromide (1%) was added, in order to visualise DNA under ultraviolet light. Gels were cast in 10 cm<sup>2</sup> Cambridge Electrophoresis (model EM 100) apparatus. Samples were mixed with 2  $\mu$ l of gel-loading buffer (Chpt 2.1.3) to increase the density of the sample and add colour for ease of loading. 1  $\mu$ l of 1Kb DNA markers (NEB) were also run for approximate size calculation. The gel was run at approximately 60 volts, 5 volts/cm (measured as the distance between electrodes).

#### **2.2.1.4 DNA purification from agarose gels**

The DNA was excised from a 1% agarose gel using a sterile scalpel under UV light. The gel slice was treated following the Qiagen protocol for QIAquick gel extraction. Briefly the gel slice was weighed and 3 volumes of Qiagen Buffer QG (Appendix C) was added and incubated at 50°C for 10 min until the agarose had dissolved. 1 volume of isopropanol was added and the DNA solution added to a QIAquick spin column and centrifuged at 13000 rpm for 1 min. The column was then washed with 0.75 ml of Buffer PE and centrifuged as before, the flow through was discarded and the column re-centrifuged as before to remove all traces of ethanol. To elute the DNA 30  $\mu$ l of water was added to the column and incubated at RT for 1 min, followed by centrifugation as before.

### **2.2.1.5 Preparation of cDNA**

Complementary DNA (cDNA) was prepared from total RNA, extracted as in Chpt 2.2.1.1, using oligo dT (Promega) in a 50  $\mu$ l reaction. 5  $\mu$ g of total RNA was diluted to a final volume of 9.5  $\mu$ l in DEPC treated water. At this stage if required RNA can be DNase treated to remove contaminating DNA by the addition of 1  $\mu$ l of RNase free DNase (HT Biotechnology) and incubation at 37°C for 10 min, followed by heat inactivation at 75°C for 5 min. 2  $\mu$ l of oligo dT 10 pM/ $\mu$ l (Promega) was added to the treated RNA and the sample incubated at 65°C for 5 min, to anneal the oligo dT to the polyA region of the RNA target, before cooling to RT. Following this incubation, 1x Reverse Transcriptase (RTase) buffer, 20 mM each of dATP, dTTP, dCTP and dGTP, 50 U of super RT (HT Biotechnology) and 50 U RNAsin (Promega) were added and incubated at 37°C for 1 hour. The synthesis was stopped by heating the reaction mixture to 94°C for 3 min. This produced a double stranded RNA/DNA hybrid for use in PCR.

### **2.2.1.6 Standard Polymerase Chain Reaction (PCR)**

PCR reactions were routinely set up in 50  $\mu$ l reaction volumes. Each reaction included 1x polymerase buffer, 2 mM magnesium chloride and 0.5 mM each dNTP, 10 pmole of each primer, 5 U of DNA polymerase, and required DNA target made up to a final volume with Millipore water. Reactions were cycled on a thermal block 94°C 5 min, followed by 30 cycles of 94°C for 1 min (denaturation), x°C for 1 min (annealing) and 72°C for 1 min 30 sec (extension) and a final 5 min extension at 72°C; where x is the

lowest theoretical annealing temperature of the primers used. PCR products were routinely cloned into pCR2.1 (Chpt 2.2.1.7).

#### **2.2.1.6.1 Addition of deoxyadenosine to the 3' ends of PCR products**

In a 30 µl reaction volume, 23 µl of PCR product was added to 25 mM MgCl<sub>2</sub>, 5 mM dNTPs, 10 x Taq reaction buffer and 1U Taq DNA polymerase and incubated at 72°C for 10 min, the product was used immediately for TA cloning (Chpt 2.2.1.7).

#### **2.2.1.7 TA Cloning (Invitrogen)**

PCR fragments were cloned into the plasmid pCR2.1 (Appendix D4) for replication in bacteria and subsequent manipulation, using the TA cloning kit (Invitrogen), following the manufacturers protocol. If a proofreading polymerase had been used for PCR, adenosine was first added to the 3' ends of the product (Chpt 2.2.1.6.1).

PCR products were ligated into 50 ng pCR2.1 TA vector according to the equation below:

$$\frac{\text{size of insert (bp)}}{\text{size of vector (bp)}} \times \text{amount of vector (ng)} = \text{amount of insert (ng)}$$

#### **2.2.1.7.1 DNA Ligation Reaction**

The required amount (vector:insert ratios of 1:1 and 1:3) of PCR product or DNA was ligated into the appropriate plasmid. Briefly 400 U of T4 DNA ligase (NEB), 1x T4 DNA ligase buffer, approximate amounts of cut vector (approx 50 ng) and insert (Chpt 2.2.1.7) were mixed with water to a final volume of 10  $\mu$ l and incubated overnight (O/N) at 14°C. Controls were set up using vector only and vector/enzyme ligations to assess digestion efficiency and background religation of the plasmid.

#### **2.2.1.8 Preparation of Electrocompetent Bacteria**

Bacterial colonies (TOP10) were maintained on minimal agar plates with no antibiotic. A single colony was picked into 10 ml of 2xTY in a 50 ml tube and grown O/N (16-20 hrs) at 37°C with shaking (225 rpm). Subsequently two sterile 2 L conical flasks each containing 500 ml 2xTY were inoculated with 5 ml of the overnight culture and grown at 37°C with shaking to an OD at 600 nm of 0.5-0.6 (approx. 2-3 hrs). The cultures were chilled on ice for 30 min and then centrifuged at 3700 g for 20 min (Beckman Avanti J25-I) at 4°C. All subsequent steps were performed at 4°C. Supernatants were decanted and the cell pellet from each flask resuspended in 500 ml of cold 1 mM HEPES (Sigma). After centrifugation as above the pellets were pooled and resuspended in a total of 500 ml 1 mM HEPES. The pooled sample was centrifuged and resuspended in 20 ml of 10% glycerol/1 mM HEPES and transferred to a 50 ml tube before further centrifugation at 4100 rpm for 20 min (ALC PK131R) at 4°C.



Finally, the pellet was resuspended in 10% glycerol, and aliquoted into pre-chilled (dry ice) 1.5 ml Eppendorfs. Aliquots were stored at -70°C until required.

#### **2.2.1.9 Transformation of Electrocompetent Bacteria**

0.5-1  $\mu$ l of ligation reaction or plasmid DNA was used to transform a 50  $\mu$ l aliquot of previously prepared electrocompetent bacteria. DNA and bacteria were mixed in a pre-chilled 1.5 ml Eppendorf (wet ice) and subsequently transferred to a 0.2  $\mu$ m electroporation cuvette (Equibio) on ice. Electroporation settings for bacterial electroporation were 2500 volts, 25  $\mu$ F, and 201  $\Omega$  using an Easyject plus electroporator (Equibio). Transformations were repeated if time constants were below 4.0 msec. Following electroporation the bacteria was transferred from the cuvette into a sterile 15 ml snap top tube with 1 ml of 2xTY. This culture was incubated at 37°C with shaking for 1hr and subsequently 50-200  $\mu$ l was plated onto 2xTY plates with the appropriate antibiotic selection. The plates were incubated overnight in a 37°C incubator.

#### **2.2.1.10 Glycerol Stocks**

A 3 ml culture (2xTY and appropriate antibiotic) was inoculated with a single bacterial colony, and grown O/N at 37°C with shaking. 700  $\mu$ l of the culture was aliquoted into pre-labelled 1.5 ml Eppendorf tubes containing 300  $\mu$ l of sterile 50% glycerol. The solution was mixed and snap frozen on dry ice before transferring to a -70°C freezer.

### **2.2.1.11 PCR Screening of bacterial colonies**

10 to 20 bacterial colonies were routinely screened for gene insertion into the vector backbone. The PCR reaction was performed in 10  $\mu$ l reaction volumes, for 10 reactions a stock of PCR reaction mix was prepared as follows:

10  $\mu$ l 10 x PCR buffer

10  $\mu$ l dNTP mix (5mM each)

1  $\mu$ l forward primer (10 $\mu$ M)

1  $\mu$ l reverse primer (10 $\mu$ M)

10  $\mu$ l MgCl<sub>2</sub> (25mM)

1  $\mu$ l Taq polymerase (Promega)

Water to 100  $\mu$ l

A single colony was picked from a 2xTY plate using a 1  $\mu$ l inoculating loop (NUNC) into a 10  $\mu$ l aliquot of reaction mix followed by streaking onto a fresh 2xTY plate. PCR reactions were cycled on a thermal block, 95°C 1 min, 45°C 1 min, and 72°C 1 min for 30 cycles. 4  $\mu$ l of each reaction was run on an agarose gel (Chpt 2.2.1.3). The 2xTY plates were incubated O/N at 37°C.

### **2.2.1.12 Preparation/purification of plasmid DNA**

A single colony from an O/N 2xTY plate was used to inoculate 2xTY. The liquid cultures were grown O/N at 37°C with shaking.

The plasmid DNA was isolated from the bacteria using Qiagen column anion exchange resin following manufacturers instructions.

A Qiagen Miniprep kit (yielding up to 20  $\mu$ g plasmid DNA) was routinely used to prepare DNA for cloning. For larger amounts of DNA either a Qiagen Midiprep (yielding up to 100  $\mu$ g of plasmid DNA) or Qiagen Mega/Gigaprep (yielding up to 2.5/10 mg of plasmid DNA) was used. Composition of buffers can be found in Appendix C.

#### **2.2.1.13 Ethanol precipitation**

To the resuspended DNA three volumes of 70% ethanol ( $-20^{\circ}\text{C}$ ) and 3 M sodium acetate pH 5.2 to a final concentration of 0.3 M was added, mixed vigorously and incubated at  $-20^{\circ}\text{C}$  for a minimum of 2 hours. The DNA was centrifuged at 10000 rpm for 15 min and the pellet washed with 70% ethanol and centrifuged as before. The DNA pellet was air dried at RT for 5-10 min. The DNA can then be resuspended in the appropriate volume of water or sterile saline.

#### **2.2.1.14 DNA Sequencing**

Sequencing reactions were set up with big Dye terminator v1.1 cycle sequencing kit (Applied Biosystems). Reactions were set up in 10  $\mu$ l reaction volumes. Each reaction included 2  $\mu$ l of pre-mix (containing polymerase and dNTP flurochromes), 1x sequencing buffer, 0.16 pmol primer and 100 ng of plasmid DNA or 10 ng of PCR product. Reactions were cycled on a thermal block for 25 cycles of  $96^{\circ}\text{C}$  10 sec,  $48^{\circ}\text{C}$ ,

5 sec, 60°C, 4 min. Samples were precipitated by vigorous mixing of the reaction with 1  $\mu$ l of 3 M sodium acetate pH 5.2 in a 1.5 ml Eppendorf tube, before the addition of 25  $\mu$ l of 100% ethanol and incubation at RT for 15 min. The precipitated DNA was harvested by centrifugation at 15000 g for 20 min and the pellet was then washed with 0.2 ml of 70% ethanol and air dried for approx. 10 min. Pellets were resuspended in 10  $\mu$ l of HiDi formamide (Applied Biosystems) and transferred to a 96 well plate (Applied Biosystems). All sequencing was undertaken in the Division of Transfusion Medicine on a 3100 ABI prism genetic analyzer following manufacturers instructions.

#### **2.2.1.15 Restriction Enzyme Digest**

Restriction digests were routinely performed at the temperature recommended by the supplier for 1.5-4 hours. To an appropriate amount of DNA in water, 1/10th of the volume of the recommended buffer and 1/10th volume of enzyme were added. For double digests the total amount of enzyme did not exceed 1/10th of the final volume to avoid non-specific activity. Following digestion fragments were visualised on a 1% agarose gel. Appropriate fragments were purified using Qiagen Qiaquick gel extraction kit (Chpt 2.2.1.4).

#### **2.2.1.16 Filling of 5' overhangs (Polymerase reaction)**

To an appropriate amount of DNA with restriction enzyme cut overhangs, 3 U T4 DNA polymerase (NEB), 1 x T4 DNA polymerase buffer, and a final concentration of 1.25 mM dNTP's was added and made up to a final volume of 50  $\mu$ l with water. The

reaction was incubated at 37°C for 30-60 min. Samples were heat inactivated at 75°C for 10 min.

#### **2.2.1.17 Site Directed Mutagenesis**

Mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene). Oligos were designed to anneal to the same sequence on opposite strands of the plasmid and incorporate the desired mutation. Oligos were between 25 and 45 nucleotides long with a melting temperature of  $\geq 78^{\circ}\text{C}$ . PCR was performed to amplify the entire plasmid using 10 ng template in pMT/BiP/CaM, 1x reaction buffer, 125 ng of each oligo, 1  $\mu\text{l}$  dNTP mix, 3  $\mu\text{l}$  Quik solution and 1  $\mu\text{l}$  *PfuTurbo* DNA polymerase in a 50  $\mu\text{l}$  reaction. Reactions were cycled on a thermal block 95°C 1 min, followed by 18 cycles of 95°C for 50 sec (denaturation), 60°C for 50 sec (annealing) and 68°C for 1 min/kb of plasmid length (extension) and a final 7 min extension at 68°C. 1  $\mu\text{l}$  of DpnI was added to the reactions and incubated at 37°C for 1 hour to digest the parental DNA. 1  $\mu\text{l}$  of the digested template was used to transform electrocompetent TOP10 bacteria (Chpt 2.2.1.9). Minipreps (Chpt 2.2.1.12) were made from the resulting colonies and sequenced (Chpt 2.2.1.14) to confirm nucleotide substitution.

| Restriction<br>Endonuclease   | Restriction Site               | NEB reaction<br>buffer | Reaction<br>Temperature (°C) |
|-------------------------------|--------------------------------|------------------------|------------------------------|
| ApaL I                        | GTGCAC<br>CACGTG               | NEBuffer 4             | 37                           |
| BglII                         | AGATCT<br>TCTAGA               | NEBuffer3              | 37                           |
| DpnI<br>(methylated DNA only) | GATC<br>CTAG                   | NEBuffer 4             | 37                           |
| Hind III                      | AAGCTT<br>TTCGAA               | NEBuffer 2             | 37                           |
| Nco I                         | CCATGG<br>GGTACC               | NEBuffer 4             | 37                           |
| Not I                         | GCGGCCGC<br>CGCCGGCG           | NEBuffer 3             | 37                           |
| Sac I                         | GAGCTC<br>CTCGAG               | NEBuffer 1             | 37                           |
| Sal I                         | GTCGAC<br>CAGCTG               | NEBuffer 3             | 37                           |
| Sfi I                         | GGCCNNNNNGGCC<br>CCGGNNNNNCCGG | NEBuffer 2             | 50                           |
| Xho I                         | CTCGAG<br>GAGCTC               | NEBuffer 2             | 37                           |

Table 2.2 Commonly used restriction endonucleases. The DNA recognition sequence for each enzyme is shown with red type distinguishing the overhangs generated. N is any base. The optimum reaction buffers and temperatures are shown for each enzyme.

## **2.2.2 Recombinant Protein Expression**

### **2.2.2.1 Maintenance of Schneider 2 (S2) cell line**

S2 cells were maintained in Schneiders *Drosophila* medium (Gibco), supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) (Gibco) at 26°C (complete media). Cells were cultured in sterile conditions.

### **2.2.2.2 Calcium Phosphate Transfection of S2 cells**

On the day of transfection  $3 \times 10^6$  S2 cells were seeded in 3 ml of complete media into each well of a 6 well plate (Costar), and incubated at 26°C for approx. 9 hours until they reached a density of  $2 \times 10^6$ /ml. Plasmid DNA was prepared using Qiagen midiprep kit and resuspended in Millipore water to approx  $2 \mu\text{g}/\mu\text{l}$ . For each transfection 2 tubes were prepared one with 300  $\mu\text{l}$  of 2xHEPES buffered saline (HBS) and one with 19  $\mu\text{g}$  DNA, 0.24 M calcium chloride, and tissue culture grade water to 300  $\mu\text{l}$ . Over a gentle vortex the DNA containing mixture was slowly titrated into the HBS, followed by incubation for 40 min at RT. The calcium phosphate DNA mixture, which should be cloudy in appearance, was slowly added drop wise to the cells, which were subsequently incubated overnight at 26°C. At 16-24 hour post-transfection the cells were washed three times with complete media and resuspended in 3 ml of fresh complete media. Expression was induced by the addition of copper sulphate to a final concentration of 500  $\mu\text{M}$ ; supernatants and cells were harvested 5-7 days post-

induction. The cell pellet and supernatant were assayed for the presence of the protein by Western blot (Chpt 2.2.2.8.2).

### **2.2.2.3 Stable Transfection of S2 cells**

Cells were treated as for transient transfection except 1  $\mu$ g of pCoBlast vector (Invitrogen) was co-transfected with the cloned pMT plasmid. This vector confers resistance to Blasticidin. Stable cell lines were not induced but were incubated for a further 2 days and then selected for resistance to blasticidin by the addition of 25  $\mu$ g/ml of Blasticidin for 2 weeks. During selection cells were washed every 5 days into 3 ml of complete media with selection and replated into the same wells. After the selection period cells were split 1:2 into a new 6 well plate and half induced as before to check for expression of the protein. Once expression was confirmed cells were grown up for large-scale production in Ex-Cell 420 serum free media (JRH Biosciences, Kansas, USA). Cells were passaged in serum free media to a final density of  $2-4 \times 10^6$ /ml; this process was repeated until a large volume was reached (usually approx. 200ml). Once the required volume was obtained the cells were incubated O/N at 26°C with shaking (100 rpm) and then protein expression was induced by the addition of 500  $\mu$ M copper sulphate. Cells were grown for a further 5-7 days following which the supernatant was harvested, cleared by centrifugation at 18000 g for 20 min at 4°C and then passed through a 2  $\mu$ m stericup filter (Millipore).



#### 2.2.2.4 Purification of Calmodulin tagged proteins expressed from S2 cells

To purify calmodulin tagged proteins NHS-activated HiTrap columns (Amersham Pharmacia Biotech) were prepared using N-(6-Aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) (Sigma)(Figure 2.1).

#### 2.2.2.4.1 Preparation of W7 column

W7 resin is a chemical compound, which has been shown to bind specifically to the hydrophobic pocket of calmodulin, in the presence of calcium, via the naphthalene ring (Osawa *et al.*, 1999).

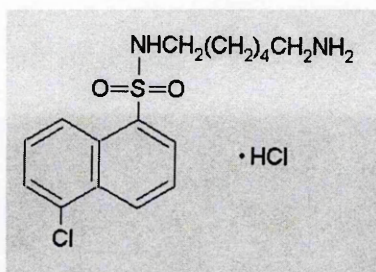


Figure 2.1 – Chemical structure of N-(6-Aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7).

A 5 mM solution of W7 was prepared by dissolving 20 mg of W7 in 20 ml of 19:1 ethanol: isopropanol mix. The W7 was allowed to dissolve overnight at RT before being mixed with an equal volume of 50 mM borate buffer pH 8.3-5 whilst vortexing to ensure complete mixing. This yielded a clear solution. A 1 ml NHS-activated HiTrap column was connected to a peristaltic pump and the storage buffer washed out using 10 ml freshly prepared 1 mM HCl at a flow rate of 1 ml/min. 10 ml of W7/

Borate solution was then recycled through the column for at least 3 hours (or O/N). The column was washed with 10 ml 70% ethanol and then blocked with equilibration buffer (0.5 M NaCl, 50 mM Tris, 1 mM  $\text{CaCl}_2$ ) at a flow rate of 1 ml/min for 30 min. Finally, 10 ml of elution buffer (0.15 M NaCl, 50 mM Tris, 10 mM EDTA) followed by 10 ml of equilibration buffer were passed over the column. Columns were stored closed at 4°C in elution buffer. Before use stored columns were re-equilibrated by washing out the elution buffer with 10 ml of equilibration buffer. All steps were performed at 4°C.

#### **2.2.2.5 Preparation of supernatants and purification of CaM tagged proteins**

Filtered supernatants were supplemented with NaCl, Tris and  $\text{CaCl}_2$  to final concentrations of 0.3 M, 50 mM, and 1 mM respectively. This supplemented supernatant was loaded onto a W7 column at a flow rate of 1 ml/min in approx 200 ml batches. The column was subsequently washed with 30 ml of equilibration buffer and the purified protein eluted in 1 ml fractions with elution buffer. The major peak of the protein was eluted in fractions 2-4. The column can be re-equilibrated with equilibration buffer or stored in elution buffer. Each eluted fraction was quantified by BCA assay (Chpt 2.2.2.10), and visualised by SDS-PAGE with subsequent Coomassie staining (Chpt 2.2.2.8.1).

#### **2.2.2.6 Purification of Histidine tagged proteins expressed from S2 cells**

Histidine tagged proteins were purified using HiTrap Nickel chelating columns (Amersham Pharmacia Biotech).

#### **2.2.2.6.1 Preparation of Nickel chelating column**

For purification using HiTrap Nickel columns, a 1 ml chelating column was washed out with 5ml of distilled water, and 0.5 ml of 0.1 M Nickel sulphate was loaded at a flow rate of 1ml/min. The column was washed again with 5ml of water followed by 5 ml of 250 mM Imidazole/IMAC (elution buffer) and 5 ml of 100 mM imidazole/IMAC (equilibration buffer).

#### **2.2.2.6.2 Preparation of supernatants for purification**

Supernatants containing histidine tagged proteins were dialysed into 1 x IMAC buffer (Chpt 2.1.3) with at least 3 changes at 4°C. The dialysed supernatant was supplemented with 500 mM imidazole/1x IMAC to a final concentration of 25 mM imidazole and filtered through a 2  $\mu$ m stericup filter. Supernatants were applied to the column at a flow rate of 1 ml/min, which was then washed with 100 mM Imidazole/IMAC. Histidine tagged proteins were eluted in 1 ml fractions in 250 mM Imidazole/IMAC. 10  $\mu$ l of each fraction was run on reducing SDS-PAGE gel and Coomassie stained (Chpt 2.2.2.8.1).

#### **2.2.2.7 Preparation of conjugated calmodulin binding peptide (N9A)**

N9A peptide (CAAARWKKAFIAVSAANRFKKIS) was synthesized by C. Graham Knight (Department of Biochemistry, University of Cambridge). The synthesised N9A peptide was conjugated to BSA (Bernatowicz & Matsueda, 1986) and peroxidase (Dr

Peter Smethurst). Polymerised, maleimide-activated peroxidase (HRP; Sigma) was used for the production of HRP-N9A following manufacturers instructions. SDS-PAGE analysis of molecular weight post-conjugation indicated that 2-6 N9A molecules were conjugated per BSA molecule. 1-4 N9A molecules per HRP polymer were estimated from molar ratio of peptide to polymer used.

### **2.2.2.8 SDS PAGE gels and immunoblotting**

#### **2.2.2.8.1 Preparation, running and staining of reducing SDS PAGE gels**

SDS PAGE gels were prepared at the appropriate percentage for linear separation of proteins. 10% gels provide clear separation of proteins of 16-68 kDa, 12% proteins of 12-43 kDa (Table 2.2). Gels were stored at 4°C.

SDS PAGE gels were prepared and run using the Protean III system (Biorad).

Gels were placed in the electrode of the Protean III apparatus and 1x SDS PAGE running buffer (Chpt 2.1.3) poured into the interior and exterior chambers. Samples were diluted in an equal volume of 2x laemelli sample buffer (Chpt 2.1.3) and incubated at 99°C for 3 min before loading. SDS gels were run at 25 volts/cm for approx. 1 hour, or until the dye front reached the bottom. Gels were immunoblotted (Chpt 2.2.2.8.2) or stained with Coomassie blue (Chpt 2.1.3) followed by incubation with destain (Chpt 2.1.3) with shaking at RT.

| Component                | Percentage of gel |       |       |
|--------------------------|-------------------|-------|-------|
|                          | 5% stack          | 10%   | 12%   |
|                          |                   |       |       |
| Sterile H <sub>2</sub> O | 3.7               | 1.9   | 1.6   |
| 30% acrylamide           | 0.5               | 1.7   | 2.0   |
| 1.5 M Tris-HCl pH 8.8    | na                | 1.3   | 1.3   |
| 1.0 M Tris-HCl pH 6.8    | 0.62              | na    | na    |
| 10% SDS                  | 0.05              | 0.05  | 0.05  |
| 10% APS                  | 0.05              | 0.05  | 0.05  |
| TEMED                    | 0.005             | 0.002 | 0.002 |

Table 2.3 Recipes for making SDS-PAGE gels. The components (ml) needed to make one 10% and 12% resolving gel and 5 ml of 5% stacking gel are listed.

#### **2.2.2.8.2 Western Blot**

Western blotting was performed using a semi-dry transblot apparatus (Atto). Six-gel-sized pieces of 3 MM paper and one piece of nitrocellulose (Protran, Schleicher and Schuell, Dassel, USA) were soaked in transblot buffer for approx. 10 min prior to blotting. SDS gel and nitrocellulose were carefully sandwiched between the six pieces of 3 MM paper and placed on the transblotter with the nitrocellulose toward the positive electrode. The gel was blotted for approx. 75 min at 0.1 mA/cm<sup>2</sup>.

#### **2.2.2.8.3 Detection of Calmodulin tagged proteins by Western blot**

Following protein transfer to a nitrocellulose membrane, CaM tagged proteins were detected with HRP-N9A. Membranes were blocked for 1 hour in TBS containing 1% BSA, 0.1% Tween 20 and 1 mM CaCl<sub>2</sub> (TBS-BC). Following blocking, the membranes were incubated for 20 min at RT with HRP- N9A (0.05 µg/ml) diluted in TBS-BC. After extensive washing with TBS-BC, the binding of HRP-N9A was detected by enhanced chemiluminescence (ECL) (Chpt 2.2.2.9).

#### **2.2.2.8.4 Detection of Histidine or V5 tagged proteins by Western blot**

Following protein transfer to a nitrocellulose membrane, His tagged proteins were detected with mouse monoclonal IgG 2b anti-His HRP (Invitrogen) and the V5 epitope were detected with mouse monoclonal IgG 2b anti-V5 HRP (Invitrogen). Membranes were blocked for 15 min in PBS containing 2.5% casein and 0.05% Tween 20.

Following blocking, the membranes were incubated for 90 min at RT with the mouse monoclonal HRP, 1:5000 dilution in PBS containing 0.05% Tween (PBS-Tw). After extensive washing with PBS-Tw, the binding of the HRP antibody was detected by enhanced chemiluminescence (ECL) (Chpt 2.2.2.9).

#### **2.2.2.9 Enhanced Chemiluminescence (ECL)**

Immunoblots probed with HRP conjugated antibodies were developed using ECL detection reagents (Amersham Pharmacia Biotech, UK). Blotted and probed nitrocellulose was washed with the appropriate buffer. After final washing an equal volume of reagents 1 and 2 were mixed (total volume of 3 ml/60 cm<sup>2</sup>), gently applied to the nitrocellulose and incubated for 1min at RT, no agitation. The ECL reagent was removed and the nitrocellulose dabbed with tissue to remove excess reagent. Finally the nitrocellulose was wrapped in Saran wrap, exposed to photographic film (Fuji medical X-ray film, Japan) for between 30 sec and 5 min and developed using a RP X-OMAT processor (model M6B).

#### **2.2.2.10 Quantification of purified protein by BCA Assay**

10  $\mu$ l samples of eluted column fractions or other recombinant proteins were assayed in triplicate in 96 well plates (NUNC) using a bicinchoninic acid (BCA) assay kit (Pierce). BSA (Bovine Serum Albumin) standards of 2, 1, 0.5, 0.25, 0.125, 0.06 mg/ml were used. Elution buffer alone or sample diluent was used as a blank. BCA containing buffer was mixed with 4% cupric sulphate, 50 parts to 1, and 200  $\mu$ l was

added to each well containing 10  $\mu$ l of protein solution. The plate was incubated at 37°C for 30 min. A purple colour developed in wells containing protein, and the optical density was measured at 562 nm. Protein concentration was determined from the BSA standard curve.

### **2.2.3 Production of Immune serum**

#### **2.2.3.1 Rabbit Immunisation by i.m. needle injection**

Rabbits were immunised according to Home Office guidelines under a project license at the National Institute for Biological Standards and Control (Herts, UK). Dutch Rabbits were vaccinated with 200  $\mu$ g of pVAC2-scFv plasmid DNA in sterile PBS injected into two sites in the quadriceps muscles. i.m. injections were given at days 0, 14 and 28. Bleeds were taken at days 0 and 35 to measure antibody responses.

#### **2.2.3.2 Human Vaccination under LIFTT clinical trial**

Patients with Follicular Lymphoma enrolled on the LIFTT clinical trial were immunised with their corresponding pVAC2-scFv. The vaccine was administered at weeks 0, 1, 2, 4, 8 and 12.



## **2.2.4 ELISA Assays**

### **2.2.4.1 IgG ELISA for anti-tetanus**

Tetanus Toxoid for flocculation (NIBSC) was coated at 10 µg/ml in PBS onto a 96-well ELISA plate (NUNC maxisorp) at 37°C for 2 hours. The wells were blocked with 100 µl 2.5% BSA in PBS containing 0.05% Tween-20 at room temperature for 1 hour. Next, the wells were incubated with 100 µl of serial dilutions of anti-sera (prepared in blocking buffer). All washings were performed with 200 µl of wash buffer (PBS, 0.05% Tween). Bound immunoglobulins were detected by incubation with 100 µl of peroxidase-conjugated goat anti-rabbit IgG (Sigma) or peroxidase-conjugated goat anti-human (Jackson), 1:4000 dilution in blocking buffer and visualised using 100 µl 3,3',5, 5' tetramethylbenzidine (TMB) substrate (Sure-blue, KPL, Guilford, UK). The plates were incubated for 5 min at RT and the reaction was stopped with 25 µl 2M H<sub>2</sub>SO<sub>4</sub>. The O.D. value at 450 nm was taken for each sample (Dynatech MRX 500).

### **2.2.4.2 IgG ELISA for anti-idiotypic**

N9A cross-linked to BSA (BSA-N9A) was coated at 5 µg/ml in 0.05 M Borate onto a 96-well ELISA plate (NUNC maxisorp) at 4°C overnight. The wells were blocked with TBS containing 10% BSA and 1 mM calcium chloride (blocking buffer) at 37°C for 2 hours before the addition of 5 µg/ml of purified scFv-CaM. Next, the wells were incubated with 100 µl of serial dilutions of anti-sera, prepared in blocking buffer. All

washings were performed with 200 µl of wash buffer (TBS containing 0.1% Tween and 0.1 mM CaCl<sub>2</sub>). Bound immunoglobulins were detected as before (Chpt 2.2.4.1).

#### **2.2.4.3 Inhibition ELISA**

B2 IgG1 (gift from Dr K Armour) was coated at 10 µg/ml in PBS onto a 96-well ELISA plate (Falcon, 353912 PVC) at 37°C for 2 hours. The wells were blocked with 2.5% BSA in PBS containing 0.05% Tween-20 at room temperature for 1 hour. Prior to blocking the serum samples (at a dilution determined by a prior titration) were incubated with doubling dilutions of inhibitor (*Drosophila* and *E. coli* derived recombinant scFv) and incubated at RT for 1 hour, equal volumes of serum and inhibitor were used. A no inhibitor control was also set up, serum plus equal volume of PBS. 100 µl of the serum, inhibitor mix was added to the plate in duplicate and incubated at RT for 1 hour. All washings were performed with 200 µl of wash buffer (PBS, 0.05% Tween). Bound immunoglobulins were detected as before (Chpt 2.2.4.1).

#### **2.2.4.4 ELISA for detection of N-linked glycosylation**

N9A cross-linked to BSA (BSA-N9A) was coated at 5 µg/ml in 0.05 M Borate onto a 96-well ELISA plate (NUNC maxisorp) at 4°C overnight. The wells were blocked with TBS containing 10% BSA and 1 mM calcium chloride (blocking buffer) at 37°C for 2 hours before the addition of 5 µg/ml of purified scFv-CaM. N-linked glycosylation was detected by incubation with 1 µg/ml of HRP labelled Concanavalin A (Con A) diluted in TBS/0.1% Tween (wash buffer) and incubated for at RT for 1

hour. All washings were performed with 200 µl of wash buffer. Bound immunoglobulins were visualised as before (2.2.4.1).

### **2.2.5 Resonant Mirror Technology**

An IAsys Auto + resonant mirror biosensor (LabSystems Affinity Sensors, Cambridge, UK) was used to study the binding of purified glycoprotein IIbIIIa (GPIIbIIIa) to the purified scFv, B2-CaM fusion protein. The BSA-N9A was coupled to the carboxymethylated dextran surface of the cuvette in 50 mM sodium acetate, pH 4, according to manufacturer's instructions. This coupling allowed the capture of the CaM tagged scFv in the presence of calcium. All experiments were performed in the presence of TBS (2.1.3) containing 10mM calcium and 0.1% Tween (wash buffer). Two alleloforms of purified GPIIbIIIa were added to the cuvette, HPA-1a1a and -1b1b. The binding of the sample was measured in arc seconds after repeated washes with wash buffer. Regeneration of the cuvette was performed by the addition of 20 mM EDTA.

### **2.2.6 Platelet Immunofluorescence Test (PIFT)**

#### **2.2.6.1 *Drosophila* supernatant**

$1 \times 10^7$  platelets in 50 µl wash buffer (0.25% BSA, 0.01 M EDTA, pH 6.8 in PBS, filtered) were incubated with 50 µl of *Drosophila* supernatant containing scFv for 1 hour at RT, the samples were washed with 4 ml wash buffer and centrifuged at 1200 g for 6 min, repeated three times. The pellet was then resuspended in 100 µl of mouse monoclonal IgG2b anti-His (Invitrogen), diluted 1:100 and incubated at RT for 1 hour. The samples were washed as before and the pellet resuspended in 100 µl of

fluorescein isothiocyanate (FITC) -conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulins (Dako), 1:20 dilution and incubated at RT for 30 min, the samples were washed as before and the pellet resuspended in 500 µl of wash buffer. All dilutions were performed in wash buffer. The samples were then analysed using a Beckman-Coulter flow cytometer.

#### **2.2.6.2 Human plasma**

Plasma collected with both EDTA and citrate was tested for platelet antibodies by the indirect PIFT using a panel of HPA genotyped donor platelets. Briefly, platelets were incubated with the patients' plasma then washed and resuspended with FITC-conjugated isotype and light chain specific conjugates (Dako Cytomation, Ely, UK). Normal human group AB plasma from a non-transfused male was used as a negative control. For investigation of temperature dependence all reagents and samples were brought to a temperature of 4, 18, 25, 30 or 37°C prior to commencing the assay. These temperatures were maintained at all stages of the assay until immediately before examination for bound FITC by flow cytometry. The fluorescence for the negative control (FITC-conjugated antibody alone) obtained with each sample was subtracted from that obtained with each test antibody to give the corrected MFI (MFI Corr).

## Chapter 3

### Recombinant scFv expression in *Drosophila* S2 Cells

#### 3.1 Introduction

Human antibody molecules and their fragments are increasingly being exploited for various scientific and clinical applications. One of the limitations to the widespread use of these technologies is the ability to express large amounts of active protein (Verma *et al.*, 1998). An ideal expression system would provide milligram quantities of pure protein without the need for time-consuming downstream processing such as multiple rounds of purification, re-folding and dialysis. Furthermore, such a system needs to be cheap, easy to manipulate and produce active recombinant protein from the majority of sources with little modification.

A Cancer Research UK (CR-UK) clinical trial has been completed using DNA vaccines encoding the single chain variable domain antibody fragment (scFv) derived from the surface immunoglobulin (sIg) of patients with low-grade follicular lymphoma (LIFTT) (CRC code: 98/04, protocol No: PH2/039). Serum samples taken from the patients pre- and post-vaccination need to be analysed for the presence of antibodies against the scFv, anti-idiotypic response. In order to measure this response a source of antigen is required. Although it is possible to isolate the antigen (sIg) from the B-cells (Kwak *et al.*, 1992) the yields are poor. Recombinant protein provides an excellent alternative, providing a reliable expression system can be found.

The scFv comprising both variable domains is regarded as the minimal structural component of an antibody required for antigen-binding activity. Immunoglobulins express three types of antigenic determinants or epitopes; these are classified as isotypes, allotypes and idiotypes. The idiotype is a set of one or more epitopes specific to the variable regions of the immunoglobulin molecule; the idiotype can be recreated by construction of the scFv. The aim of the DNA vaccination strategy in the LIFTT clinical trial is to induce a clinically relevant immune response in the patients; this response will be measured by ELISA using recombinant scFv (Chapter 4) and by T cell proliferation assays. For a successful ELISA; firstly milligram quantities of correctly folded pure scFv need to be produced and secondly a versatile affinity tag for purification and capture of the scFvs onto the solid phase is required. Earlier studies from our laboratory have revealed that the commonly used hexa-histidine tag when fused to the carboxy terminus of the V<sub>L</sub> domain is susceptible to cleavage. This affects the overall yield, purification ability, detection and potential capture of any recombinant antibody. In this chapter studies are described on the evaluation of a robust recombinant expression system in *Drosophila* of human scFv fused to calmodulin (CaM).

### **3.1.1 Review of expression systems**

Cell based *in vitro* protein expression systems can be classified into two categories, prokaryotic, the *E. coli* systems and eukaryotic, mammalian, yeast and insect cell expression systems. Each of these systems has been used effectively to express antibody fragments, each with their own advantages and disadvantages (reviewed in Verma et al (Verma *et al.*, 1998)).

#### 3.1.1.1 *Escherichia coli* (*E. coli*)

*Escherichia coli* (*E. coli*) is probably the cheapest, fastest and most widely used expression system available to research laboratories. The cell growth is typically exponential allowing detection of expressed protein in a minimum culture time of eight hours. Relatively small amounts of DNA are needed for transfection of the bacteria and the yields are substantial. Fermentation of *E. coli* has successfully produced yields of 100-130 mg/L of active scFv (Huston *et al.*, 1995). Although the yields are substantial fermentation requires large volumes of bacterial culture, which are difficult to manipulate in average research laboratories. Other disadvantages to *E. coli* expression systems include production of inactive and insoluble protein located inside inclusion bodies; in this case protein refolding protocols must be applied, which require a functional assay in order to monitor activity. *E. coli* lacks the ability to provide the post-translational modifications found in eukaryotic cells, for example, glycosylation of the mature protein; this will have an effect on the properties and activity of some proteins. In addition *E. coli* systems are less suitable for proteins with multiple disulfide bonds and refolding of these proteins is cumbersome. Finally *E. coli* produced proteins contain high concentrations of endotoxins, which need careful removal if the recombinant proteins are to be used in cell biology assays or *in vivo* experiments.

### 3.1.1.2 Mammalian

Mammalian expression systems, for example Chinese hamster ovary (CHO) cells are a widely used system with many advantages (Geisse *et al.*, 1996). CHO cells have the required molecular machinery to synthesise active protein directly. Proteins are appropriately folded, glycosylated and secreted, the latter simplifying purification. Therefore, mammalian cells inevitably produce the best materials for almost all applications including human therapeutics and diagnostics (Morrison, 1992).

Mammalian hosts have limited tolerance for certain proteins especially intracellular and membrane proteins, at least 50% of chosen proteins are poorly or never expressed and are sometimes toxic to the host (Geisse *et al.*, 1996). Working with mammalian cells in culture is demanding and specialised cell culture techniques and equipment are required. Yields may be disappointing and extensive rounds of clone selection are often needed to achieve reasonable expression levels in the µg/ml range. The overall yields are reportedly not as high as from *E. coli* (Dorai *et al.*, 1994).

### 3.1.1.3 Yeast

Compared to other eukaryotic expression systems, *Pichia pastoris* offers many advantages, because it does not have the problems of endotoxin associated with bacteria or viral contamination of proteins produced in animal cell culture. Yeast can provide some post translational modification, although not as advanced as mammalian cells. *Pichia* can be grown rapidly in simple growth medium and it has been



demonstrated that yeast is capable of producing higher levels of recombinant antibody fragments than *E. coli* (Eldin *et al.*, 1997 and Ridder, 1995 #28).

#### 3.1.1.4 Insect

Insect cells can easily be maintained in suspension cultures at room temperature, they can produce higher levels of protein than both mammalian and *E. coli* expression systems and most of the post-translational modifications used by higher eukaryotes are present (Miller, 1988; Maeda, 1989). The most common insect expression system uses baculovirus to infect insect cells of the order *Lepidoptera*, for example ovarian cell lines from *Spodoptera frugiperda* (Sf9, Sf21). Functionally active antibody fragments have been produced in this system (Hasemann & Capra, 1990; zu Putlitz *et al.*, 1990). The N-linked glycosylation sites are the same as in mammalian cells but they differ in the nature of the oligosaccharide chains, insect cells are not capable of processing mature oligosaccharides to the forms found in mammalian cells (Hsieh & Robbins, 1984). Baculovirus have a highly restricted host range and are therefore considered safer than mammalian expression systems for use in the laboratory (Groner, 1986). Disadvantages include the maintenance of a viral stock, which is time consuming; expression is under the control of a very late viral promoter such that expression peaks when the cells are dying. The inherent problems with this include no stable cell lines and the possibility of proteolysis occurring as the protein is expressed.

#### **3.1.1.4.1 *Drosophila* Expression System**

An alternative insect cell system is the *Drosophila* expression system which uses a cell line derived from a primary culture of late stage *Drosophila melanogaster* embryos, Schneider 2 (S2) cells (Schneider, 1972) and a simple plasmid vector. The *Drosophila* system has the same advantages as baculovirus but expression can be controlled using an inducible promoter. Several groups have shown that high-level transcription can be achieved with a metallothionein promoter when induced by heavy metal such as cadmium and copper (Maroni *et al.*, 1986; Otto *et al.*, 1987). Co-transfection of a second plasmid carrying a gene conferring resistance to antibiotics like hygromycin or blasticidin provides a simple method of selection (van der Straten, 1989) of stable cell lines in two weeks. Surprisingly this system has not been widely used although reports indicate good yields of a variety of proteins (Culp *et al.*, 1991; Johanson *et al.*, 1995; Kirkpatrick *et al.*, 1995; Li *et al.*, 1996).

#### **3.1.2 Glycosylation of Human Immunoglobulin G (IgG) and scFv**

Higher eukaryotes perform a variety of post-translational modifications, including methylation, sulfation, phosphorylation, lipid addition, and glycosylation. Such modifications may be of critical importance to the function of an expressed protein. Secreted proteins, membrane proteins, and proteins targeted to vesicles or certain intracellular organelles are likely to be glycosylated. The most common and best studied is N-linked glycosylation, where oligosaccharides are uniquely added to specific asparagine residues. The tripeptide sequence (or sequon), asparagine-X-

serine/threonine (Asn-X-Ser/Thr) is generally required for N-linked glycosylation (Marshall, 1974). In this sequon, X can be any amino acid, although proline, glutamic acid and aspartic acid are rarely found at this position (Marshall, 1972, 1974; Mononen & Karjalainen, 1984). Only rare examples of N-linked glycosylation at other sequences (eg Asn-X-Cys and Asn-Gly-Gly-Thr) have been described (Gavel & von Heijne, 1990). One of the major differences between mammalian and insect expression systems is the types of glycosylation added. Mammalian cells add more complex, branched glycan moieties, which are composed of mannose, galactose, N-acetylglucosamine (GlcNAc) and neuramic acids (Kornfeld & Kornfeld, 1985). Insect cell glycosylation is generally of the high-mannose type as they are not trimmed and sialylated. Human IgG is glycosylated on asparagine residue 297, on each of the CH<sub>2</sub> domains and this residue is conserved in all of the IgG subclasses. Glycosylation within the variable domains is rare, of the 52 human germline V<sub>H</sub> genes; only three encode a naturally occurring glycosylation site (V<sub>1-08</sub>, V<sub>4-34</sub> and V<sub>5a</sub>) (Zhu *et al.*, 2002). Interestingly the V domains expressed on the membrane of the malignant B cells in patients with low-grade FL are frequently glycosylated. A recent study has shown that glycosylation sites are present in the immunoglobulin V domains of 79% of patients with FL compared with 9% of B cells in healthy individuals (Zhu *et al.*, 2002). Studies of monoclonal immunoglobulins have shown that binding to antigen can be increased (Wallick *et al.*, 1988; Leibiger *et al.*, 1999) or decreased (Fujimura *et al.*, 2000) by the presence of a carbohydrate moiety in the V region. The question whether naturally occurring IgG antibodies against the less complex glycosylation moieties of the expressed scFv masks the specific binding of the human anti-idiotypic IgG to the recombinant scFv is addressed in chapter 4 of this thesis.

### 3.1.3 Tagging of recombinant proteins

For the purposes of detection, purification and immobilization tagging of recombinant proteins is important. The size of the tag can vary considerably from a small number of amino acids to complete independently folded protein domains. The selection of the tag is dependant on the functional requirements and the expression systems (Terpe, 2003). scFv were initially tagged with the c-myc tag (Ward *et al.*, 1989) and the monoclonal antibody 9E10 has been widely used for scFv detection. Purification of scFvs using affinity chromatography with 9E10 columns (Evan *et al.*, 1985) has proven cumbersome and results in poor yields. The hexa-histidine (His) tag is increasingly being selected as an alternative tag for purification. However, in the context of scFvs the His tag does have some drawbacks. Firstly the His tag is susceptible to cleavage. Secondly the nickel agarose columns used for purification are costly. Finally the purification is not single step, requiring pre and post purification dialysis substantially reducing the yields.

### 3.1.4 Calmodulin

Calmodulin (CaM) has been described as a tag for bacterial expression of scFvs (Neri *et al.*, 1995a; Melkko & Neri, 2003) with the result of better yields and no cleavage of the tag. In this chapter I describe its use in an insect cell system. Calmodulin is a ubiquitous, calcium-binding protein that can bind to and regulate a multitude of different protein targets, thereby affecting many different cellular functions. It is abundant in the cytoplasm of all higher cells and has been highly conserved during evolution. It is a small (17 kDa) dumbbell-shaped protein composed of two globular

domains connected by a flexible linker (Chattopadhyaya *et al.*, 1992). Each globular domain has two calcium binding sites. In the presence of calcium the molecule adopts a constrained configuration and in the absence of calcium it adopts a more elongated and relaxed state (Figure 3.1).

Calmodulin (CaM) is a crucial intracellular calcium regulated switch and a substantial number of intracellular proteins such as kinases and phosphatases have CaM binding domains. The CaM binding protein is trapped between each domain; this binding is calcium dependant and high affinity ( $K_a > 10^9$ ). The CaM binding domain of myosin light-chain kinase was used as a template to engineer a short oligopeptide with high affinity for CaM in the presence of calcium and no affinity in the absence of calcium. The NMR structure of the complex between calcium-bound CaM and a 26-residue synthetic peptide comprising the CaM binding domain (residues 577 to 602) of rabbit skeletal muscle myosin light chain kinase has shown that only amino acids 580 to 598 of the myosin light-chain kinase, sequence RWKKNFIAVSAANRFKKIS contact CaM (Ikura *et al.*, 1992). Alanine mutant scanning was applied to this oligopeptide extended at the amino terminus producing the 23-mer CAAARWKKAFFIAVSAANRFKKIS (N9A mutant) that showed surprisingly higher affinity ( $K_a 10^{12}$ ) for CaM than the wild type (Montigiani *et al.*, 1996).

In our laboratory this CaM was used for the expression and purification of the leucine rich repeat domain of glycoprotein (GP) Iba in Baculovirus (Li *et al.*, 2001) and the two Ig-like domains of platelet GPVI (Smethurst *et al.*, 2004). Making use of the high affinity binding of calmodulin to the organic compound

N-(6-Aminohexyl)-5-Chloro-1-Naphthalenesulfonamide (W7) a simple one step purification removes all contaminating proteins enabling the CaM tagged scFvs to be eluted under mild conditions by calcium chelation with 10 mM EDTA. Not only is the

CaM an excellent tag for purification of the recombinant protein but it also provides a reusable method for capture and immobilization of the protein onto a solid surface. For immunoassays (eg ELISA) or protein interaction studies using mirror resonance technology. The 23-mer alanine mutant (N9A) is linked to BSA and bound directly to the solid phase allowing the fusion protein to be directly captured by means of the CaM tag (Smethurst *et al.*, 2004). This enables orientation of the protein of interest with optimal exposure of the epitopes and excellent access to the active site.

### 3.1.5 Model scFv

scFv-B2 is a human anti-GPIIbIIIa specific for the leucine 33 alleleform of the  $\beta 3$  integrin (Griffin & Ouwehand, 1995). The single amino acid substitution at position 33 in GPIIIa from a leucine (a alleleform) to a proline (b alleleform) is at the basis of the Human Platelet Antigen 1 (HPA-1) system (Newman *et al.*, 1989). scFv B2 will bind HPA-1a positive but not 1a negative platelets (Griffin & Ouwehand, 1995). The allele restricted specificity of scFv B2 is retained when tested with GPIIbIIIa alleleforms purified from HPA-1 genotyped platelets. B2 is a well-characterised scFv and has previously been expressed in *E. coli* with c-myc and His tags (Griffin & Ouwehand, 1995) and in a rat myeloma cell line as a recombinant IgG1 (Watkins *et al.*, 1999).

The work in this chapter compares the characteristics of scFv B2 produced in *E. coli* and in *Drosophila*. Based on these results scFvs encoded by V genes from the malignant B cells of patients with FL were expressed as CaM fused scFvs in *Drosophila*, followed by purification.

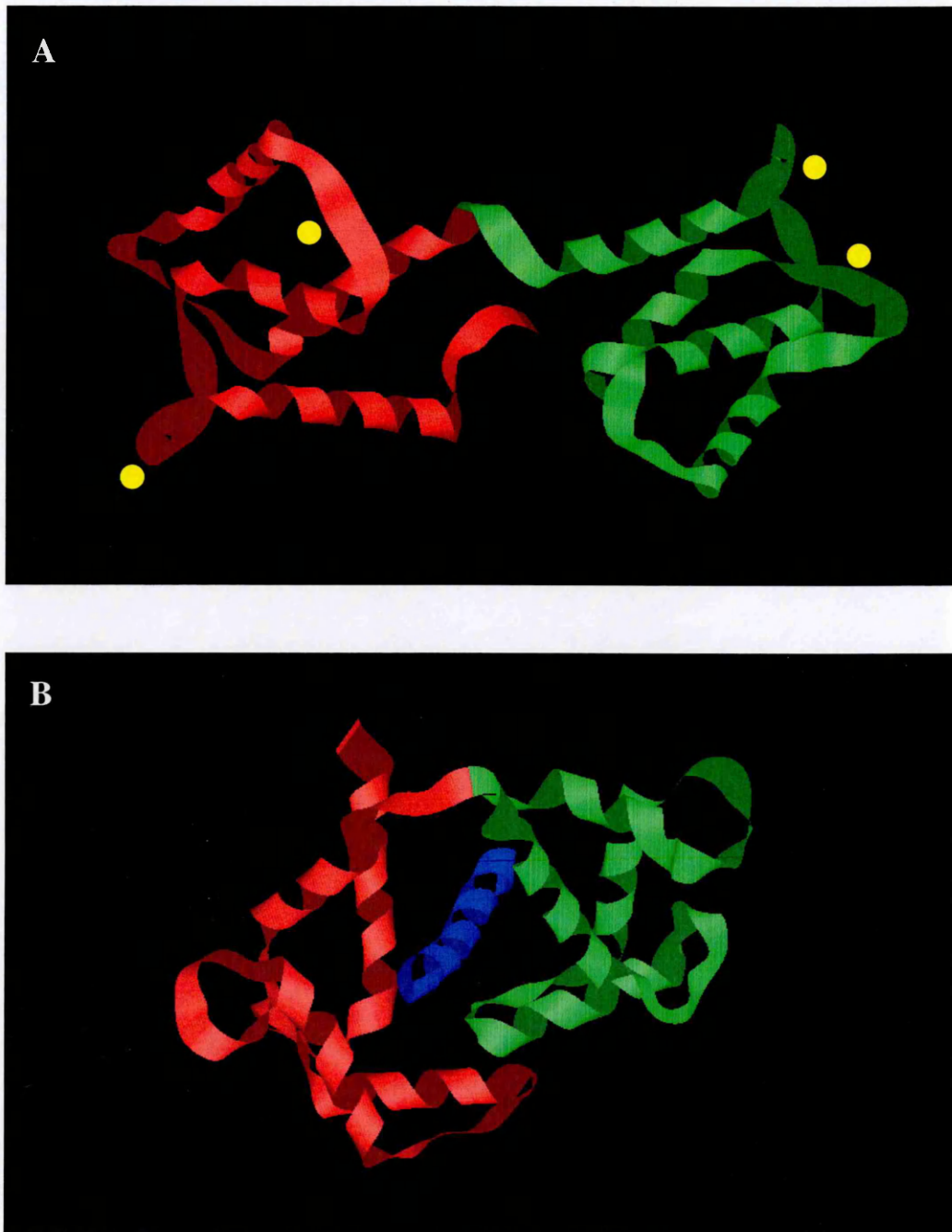


Figure 3.1 Ribbon structure of calmodulin in its resting (A) and active state (B). The four calcium-binding sites (yellow dots) are shown in A. In the absence of calcium the structure is elongated with domain 1 (red) and domain 2 (green) separated by a 28 amino acid long  $\alpha$ -helix (A). In the presence of calcium and a calmodulin binding peptide (blue) (B) the separating  $\alpha$ -helix is disrupted into two shorter helices joined by a flexible loop. The globular domains are able to come closer together forming a box like structure around the peptide. Modelled from [www.rcsb.org/pdb](http://www.rcsb.org/pdb).

## 3.2 Experimental Design

### 3.2.1 Plasmid Construction

The pMT/BiP/V5 His C inducible / secreted expression plasmid (Invitrogen) was modified in order that the cloning sites were compatible with the bacterial expression plasmids and the DNA vaccination plasmids used in our laboratory. The cloning sites are 5', NcoI, such that the ATG of the restriction site encodes for a methionine residue in the mature protein, whilst remaining in frame with the BiP signal sequence and 3', NotI, such that the affinity tags are in frame. The NotI restriction site encodes for a three-alanine spacer. pMT/BiP/V5 His C was chosen for modification as the V5 and His tags are in frame with the NotI site. The NcoI site, although already present in the multiple cloning site (MCS) of pMT/BiP/V5 His C, was in a different reading frame to our other plasmids (Figure 3.2 A). To change the reading frame of the NcoI site a double digest with BglII and NcoI (Chpt 2.2.1.15) was performed giving rise to the overhangs (Figure 3.2 B). This double digest has removed a thymine (T) from the 3' end of the BglII site and a cytosine (C) from the 5' end of the NcoI site. The NcoI site was recreated by the plasmid being re-annealed by end filling the overhangs using DNA polymerase (Chpt 2.2.1.16) followed by ligation (Chpt 2.2.1.7.1) and transformation into TOP 10 electrocompetent bacteria (Chpt 2.2.1.9). The sequence of the new cloning site is shown in Figure 3.2 C. The new plasmid pMT/BiP/V5 His D has lost the BglII site and retained the NcoI site. The removal of the two bases, C and T, has changed the reading frame so the NcoI site now encodes for methionine. (Figure 3.2 D). Cloning of gene sequences into this plasmid by means of the NcoI site add an extra four N-terminal residues, arginine, serine, methionine and alanine.



A

5'    TCG CTC GGG **AGA** TCT CCA TGG CCC GGG GTA CCT ACT    3'  
ORF1 Ser Leu Gly ▲ Arg Ser Pro Trp Pro Gly Val Pro Thr  
ORF2    Ser Arg Glu Ile Ser **Met** Ala Arg Gly Thr Tyr

Last 3 aa of BiP signal sequence BglIII site NcoI site  
Multiple cloning site

B

5'    TCG CTC GGG A            CATGG CCC GGG GTA CCT ACT    3'  
3'    AGC GAG CCC TCTAG            C GGG CCC CAT GGA TGA    5'

C

5'    TCG CTC GGG **AGA** TCC **ATG** GCC CGG GGT ACC TAC T    3'  
3'    AGC GAG CCC TCT **AGG** TAC CGG GCC CCA TGG ATG A    5'

D

5'    TCG CTC GGG **AGA** TCC **ATG** GCC CGG GGT ACC TAC T    3'  
         Ser Leu Gly ▲ Arg Ser **Met** Ala Arg Gly Thr Tyr

Last 3 aa of BiP signal sequence NcoI site

Figure 3.2 Sequential modifications to the pMT/BiP/V5 His C plasmid to obtain pMT/BiP/V5 His D. A) Nucleotide and amino acid sequence of pMT/BiP/V5 His C, open reading frame (ORF) 1 denotes the amino acid sequence of pMT/BiP/V5 His C, ORF 2 denotes the required amino acid sequence to obtain the correct ATG reading frame of NcoI (bold). The position of signal peptide cleavage is indicated▲. B) Nucleotide sequence of pMT/BiP/V5 His C after BglII and NcoI digest. C) Nucleotide sequence of pMT/BiP/V5 His D, the ATG is in bold and the bases added by DNA polymerase are in red. D) Nucleotide and amino acid sequence of pMT/BiP/V5 His D, the ATG of the NcoI site encodes for Methionine (bold) in frame with the BiP signal sequence. The position of signal peptide cleavage is indicated▲.

A 134 bp fragment containing the V5 epitope and polyhistidine tags were removed from pMT/BiP/V5 His D by restriction enzyme digest using NotI and SacI without altering any of the regulatory elements. The CaM gene was amplified by PCR using pWlba<sub>wt</sub> (Li *et al.*, 2001) as template and the primers 5'-GCC ATA GCG GCC GCT GAC CAA CTG ACA GAA GAG CA-3' and 5'-CGA CAT GAG CTC TTA TCA CTT TGA TGT CAT CAT-3', encoding NotI and SacI restriction sites respectively (underlined). Amplification was performed over 30 cycles consisting of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. The PCR product was gel purified, digested with NotI and SacI, and cloned into the NotI/SacI digested pMT/BiP/V5 His D to generate pMT/BiP/CaM (Figure 3.3).

### 3.2.2 Testing recombinant scFvs

To assess the activity of the recombinant scFv derived from expression in the S2 cells the well-characterised scFv-B2 against the leucine 33 alleloform of the  $\beta 3$  integrin was used. scFv-B2 was expressed in the *Drosophila* expression system with both the His and CaM tags and compared with recombinant B2 expressed from *E. coli* TG1 cells with a c-myc / His tag. The functional activity of B2 was defined by its ability to bind to native GPIIb/IIIa on platelets by the platelet immunofluorescence test (PIFT) (Chpt 2.2.6) and by measuring binding to purified GPIIb/IIIa by mirror resonance technology (Chpt 2.2.5).

The folding and tertiary structure of scFv-B2 was further investigated by its ability to inhibit the binding of polyclonal anti-idiotypic B2 to B2 IgG1 (Watkins *et al.*, 1999) (Chpt 2.2.4.3). The amount of protein required to inhibit the binding by 50%, the IC<sub>50</sub> value, was determined in this assay and compared between the two different His

tagged preparations. Polyclonal anti-B2 rabbit serum was produced by immunising Dutch rabbits with the DNA plasmid based vaccine pVAC2-B2 (Chpt 2.2.3.1). Further information regarding rabbit immunisations is provided in chapter 4.

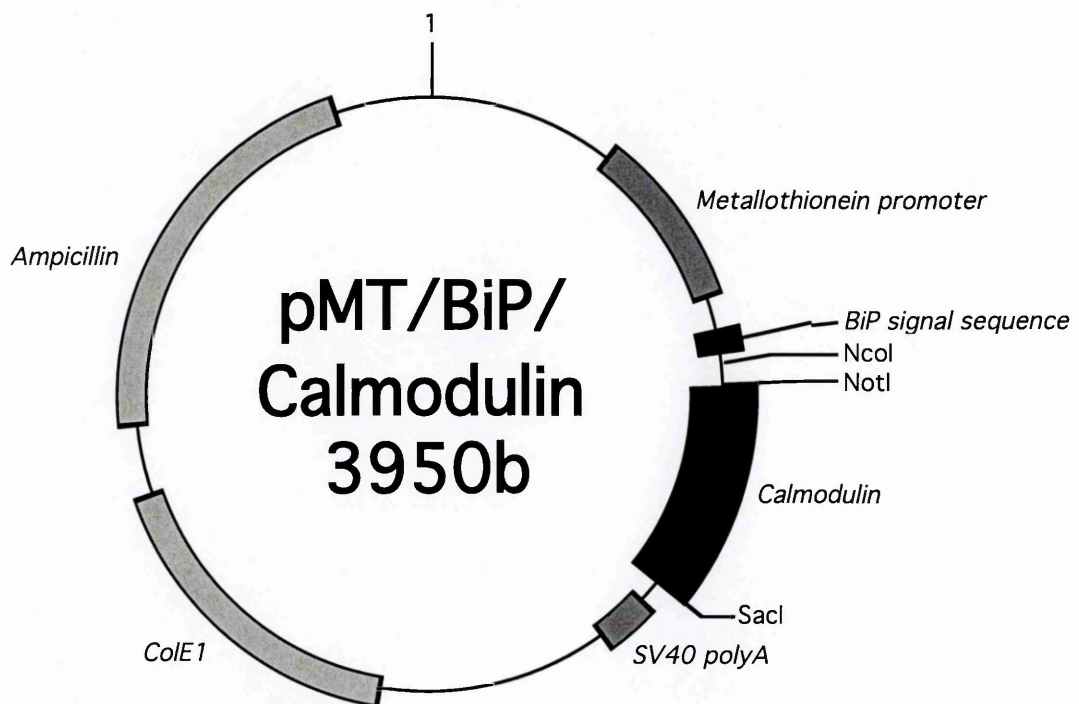


Figure 3.3 Map of pMT/BiP/CaM. Expression of the C-terminal CaM fusion protein is under the control of the metallothionein promoter. Protein secretion is directed by the signal sequence of *Drosophila* BiP protein. DNA encoding the protein of interest is cloned as an NcoI/NotI fragment.

### **3.3 Results**

#### **3.3.1 Construction of expression plasmid**

The expression plasmid pMT/BiP/V5 His D (Invitrogen) was modified by the insertion of the gene for CaM. The V5 epitope and the His-tag can be detected with polyclonal antibodies against V5 and His respectively. Proteins can only be purified via the His tag. The alternative tag for scFv was the 17 kDa protein CaM. For this the plasmid pMT/BiP/CaM was constructed and the V5/His sequence was replaced with the CaM gene sequence. The CaM gene used is derived from *Xenopus laevis* (Chien & Dawid, 1984) and encodes the same amino acid sequence as the human gene.

#### **3.3.2 Expression and characterisation of B2-His in Drosophila S2 cells**

scFv-B2 was expressed with the V5 and His tags using pMT/BiP/V5 His D. The nucleotide and amino acid sequences of B2 are in Appendix A. The level of expressed scFv in the supernatant was monitored by Western blotting over a 7-day period (Figures 3.4) following induction. scFv expression reached a maximum at day 4 and remained stable for at least 3 days (Figure 3.4). Based on these results supernatant was harvested at any day between days 4 and 7.

The S2 stable lines of scFv-B2 were expanded to a 200 ml culture in serum free media. Following a 5-day induction protein was purified from the supernatant and analyzed on a 12% SDS-PAGE gel (Figure 3.5). The recombinant protein was eluted

in fractions 2 and 3. The concentration of protein in these fractions was determined by BCA assay (Table 3.1), the overall yield of B2-His was 3 mg.

Binding of scFv-B2 His to platelets was determined by flow cytometry using HPA-1 genotyped platelets. *Drosophila* supernatant containing scFv-B2 His was incubated with platelets and bound scFv was revealed by incubation with mouse anti-His (Invitrogen), followed by goat anti-mouse immunoglobulin FITC (Dako) (Chpt 2.2.6.1). The scFv-MH11 that has activity against human factor VIII and the scFv-2F2 recognising GPIIbIIIa, but binding an epitope distinct of HPA-1a were used as negative and positive controls respectively. A median fluorescence intensity (MFI) of 20.2 and 23.3 was observed with scFv-2F2-His binding to HPA-1a1a and -1b1b platelets respectively (Figure 3.6). scFv-B2-His produced a MFI of 71.5 and 1.41 when reacted with HPA-1a1a and -1b1b platelets respectively. The latter is equivalent to the MFI of the negative control (scFv-MH11) of 1.31 and 1.39 respectively. Data shown is representative of n=2.

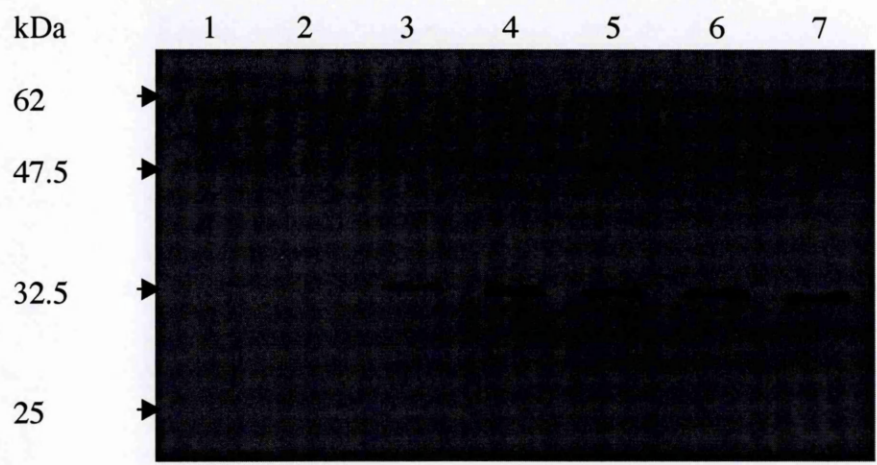


Figure 3.4 Detection of scFv-B2-His by immunoblot. A stable S2 cell line expressing B2-His was scaled up in 200 ml serum free medium before induction with 500  $\mu$ M  $\text{CuSO}_4$ . 1 ml samples were taken at days 1-7 post induction and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. His tagged proteins were detected with peroxidase labelled mouse anti-His and visualised by enhanced chemiluminescence. Lanes 1-7, days 1-7.

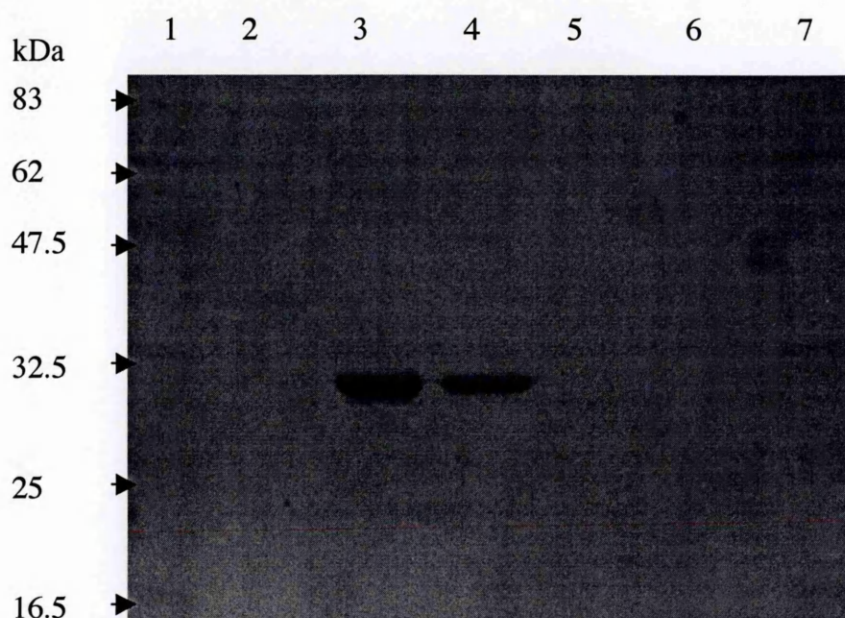


Figure 3.5 Coomassie stained SDS-PAGE gel of purified histidine (His)-tagged scFv-B2. A stable S2 cell line expressing B2-His was scaled up in 200 ml of serum free medium before induction with 500  $\mu$ M  $\text{CuSO}_4$ . On day 5 post induction the supernatant was harvested, dialysed with IMAC buffer and then supplemented with 25 mM imidazole. B2-His was purified over a Nickel chelating column and eluted in 1 ml fractions with IMAC containing 100 mM imidazole, 10  $\mu$ l of each fraction as analysed on a 12% SDS-PAGE gel and visualised with Coomassie. Lanes 1 and 7 – broad range molecular weight marker, lanes 2-6 fractions 1-5.



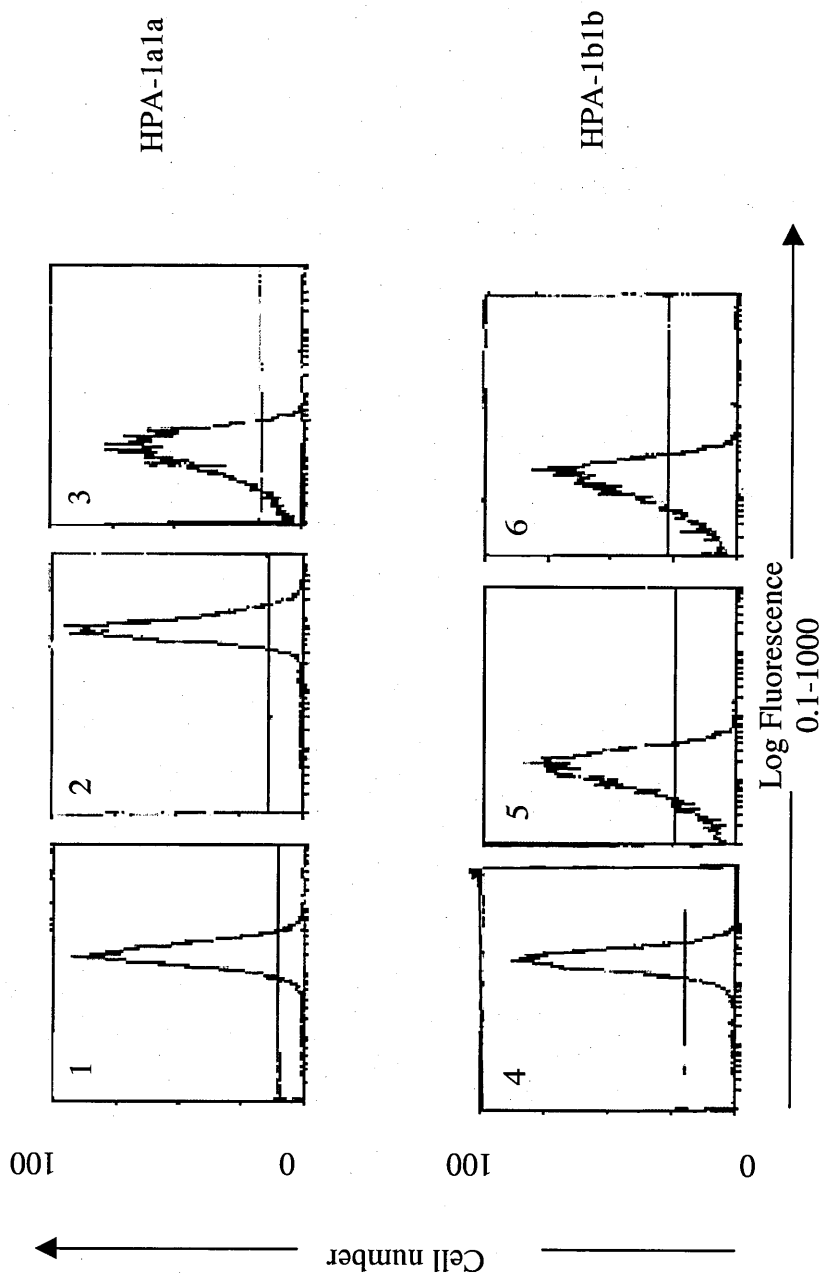


Figure 3.6 Binding of His-tagged recombinant scFv-B2 to platelets by PIFT. Supernatant from S2 cells expressing B2-His, 2F2-His and MH11-His was harvested and used in PIFT against HPA-1a1a and -1b1b platelets. Panels 1-3 binding of 2F2, B2 and MH11 scFvs to HPA-1a1a platelets. Panels 4-6 binding of 2F2, B2 and MH11 scFvs to HPA-1b1b platelets.

### 3.3.3 Comparison of B2-His expressed from *Drosophila* S2 cells and *E. coli* TG1

#### 3.3.3.1 Yields

His tagged scFv-B2 was expressed simultaneously in S2 cells and *E. coli* and purified over Ni-agarose columns. Yields of scFv after purification were determined by BCA assay. *Drosophila* supernatant produced approximately 15 mg/L of cell suspension compared with 1 mg/L of *E. coli* bacterial broth. Equal amounts of the purified scFv-B2 from both sources were analyzed on a 12% SDS-PAGE gel (Figure 3.7). scFv-B2 has an approximate molecular weight of 26 kDa, this is increased by the presence of c-terminal tags (Figure 3.7). There is also a minor difference in molecular weight between the scFv-B2 expressed from *E. coli* and that from *Drosophila* S2 cells due to differences in molecular weight of the c-terminal tags used in each system.

#### 3.3.3.2 Activity

Polyclonal rabbit antibodies against the B2 idiotype were generated by DNA based vaccination of Dutch rabbits (Chpt 2.2.3.1). The majority of antibodies present in the rabbit sera will recognise tertiary structures. Inhibiting the binding of the serum IgG antibodies to B2-IgG1 will give an idea of how well folded each protein is. The potency of His tagged scFv-B2 produced from *Drosophila* S2 cells and *E. coli* was determined by inhibiting the binding of these polyclonal rabbit antibodies to the B2 idiotype (Figure 3.8). IgG1 B2 was used as a positive control. In short, B2 IgG1 was immobilized on the solid phase and the signal of the rabbit antiserum was determined

in the presence of increasing amounts of scFv-B2 from either source. The amount of each inhibitor required to reduce the binding of the rabbit serum to B2 IgG by 50% (IC<sub>50</sub>) was extrapolated from the graph using GraphPad Prism®. The IC<sub>50</sub> value was calculated for *Drosophila* B2-His at 3.8 µg/ml, *E. coli* B2-His at 7.7 µg/ml and B2 IgG1 at 1.3 µg/ml.

### **3.3.4 Expression and validation of B2-CaM in *Drosophila* S2 cells**

scFv-B2 was expressed with a CaM tag using pMT/BiP/CaM. The level of expressed scFv in the supernatant was monitored by Western blotting over a 7-day period (Figures 3.9) following induction. scFv expression reached a maximum at days 3 to 5 (Figure 3.9). After day 5, the level appeared to reduce.

The S2 stable lines of scFv-B2 were expanded to a 200 ml culture in serum free media. Following a 5-day induction protein was purified from the supernatant and analyzed on a 12% SDS-PAGE gel (Figure 3.10). The recombinant protein was eluted in fractions 2 and 3. The concentration of protein in these fractions was determined by BCA assay (Table 3.1). The overall yield of purified B2-CaM from 200 ml of culture supernatant was 4 mg.

The CaM tagged scFv-B2 was validated for functional activity using mirror resonance technology (Figure 3.11). The CaM binding peptide N9A was immobilized onto the surface of the cuvette and scFv B2-CaM was captured on the BSA-N9A to 1200 arc seconds (Figure 3.11 A). Purified GPIIbIIIa from HPA-1a1a and -1b1b platelets was added in turn to the cuvette and binding was allowed to take place (Figure 3.11 B and

3.11 C). As expected the GPIIbIIIa from the -1a1a platelets associated strongly to scFv-B2 and on washing dissociated slowly (Figure 3.11 B). Any binding to scFv-B2 by the GPIIbIIIa from the -1b1b platelets was removed immediately after the addition of wash buffer (Figure 3.11 C).

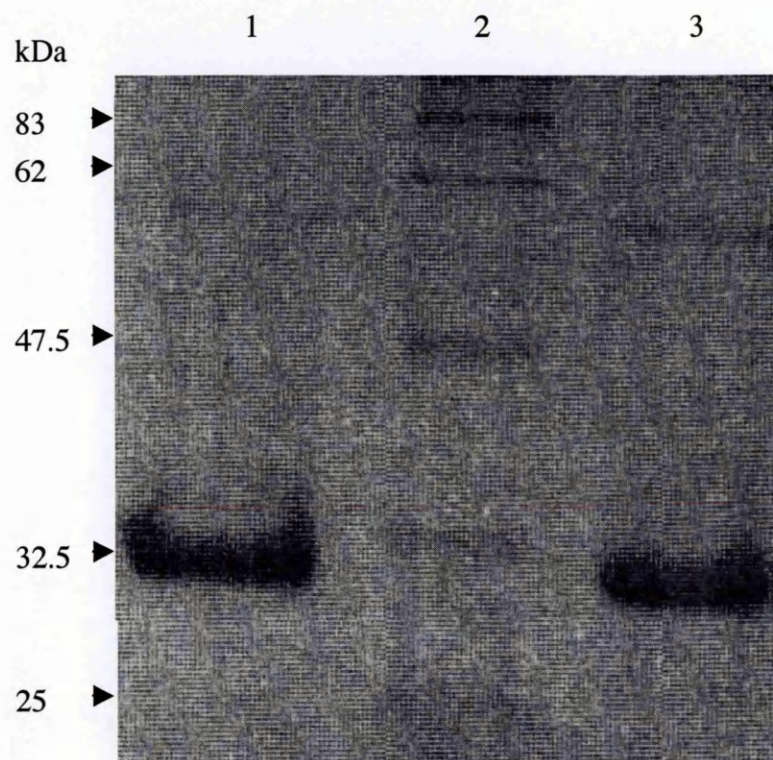


Figure 3.7 Coomassie stained SDS-PAGE gel of purified His tagged scFv-B2. A stable S2 cell line expressing B2-His was induced to express as in Figure 3.5. *E. Coli* TG1 were induced with IPTG to express B2-His. Osmotic and periplasmic preparations were isolated from the bacterial pellets and pooled. This along with the *Drosophila* supernatant were dialysed with IMAC buffer and supplemented with 25 mM imidazole. B2-His was purified over a Nickel chelating column and eluted in 1 ml fractions with IMAC containing 100 mM imidazole, 10  $\mu$ l of each fraction as analysed on a 12% SDS-PAGE gel and visualised with Coomassie. Lane 1, *Drosophila* scFv-B2-His; Lane 3 *E. coli* scFv-B2-His; Lane 2 broad range molecular weight marker.

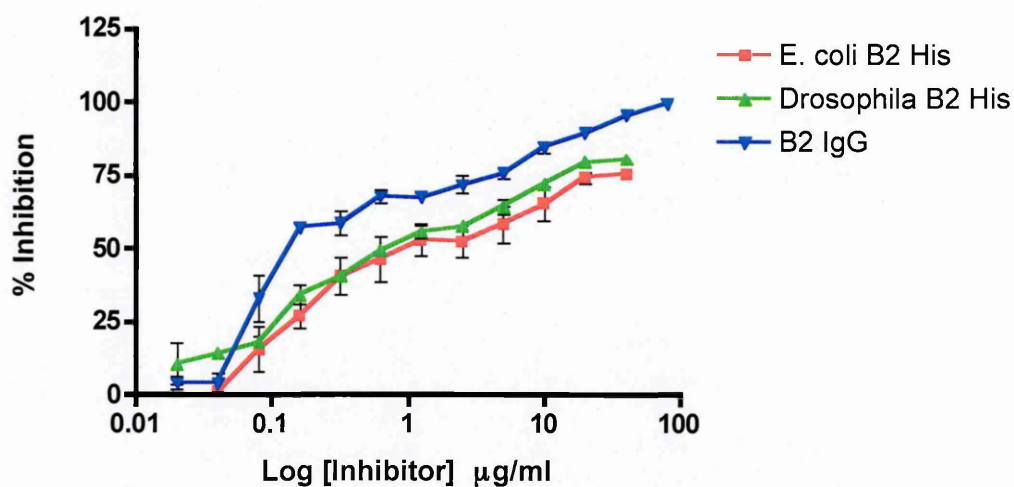


Figure 3.8 Inhibition of the binding of polyclonal anti-idiotypic B2 (rabbit sera) to B2 IgG1 with *Drosophila* S2 and *E. coli* derived B2-His and with B2 IgG1. Each line represents a titration of inhibitor. Minimum and maximum inhibition concentrations are 20 ng and 80  $\mu\text{g/ml}$  respectively. The  $\text{IC}_{50}$  values were extrapolated from the graph using GraphPad Prism<sup>®</sup>.

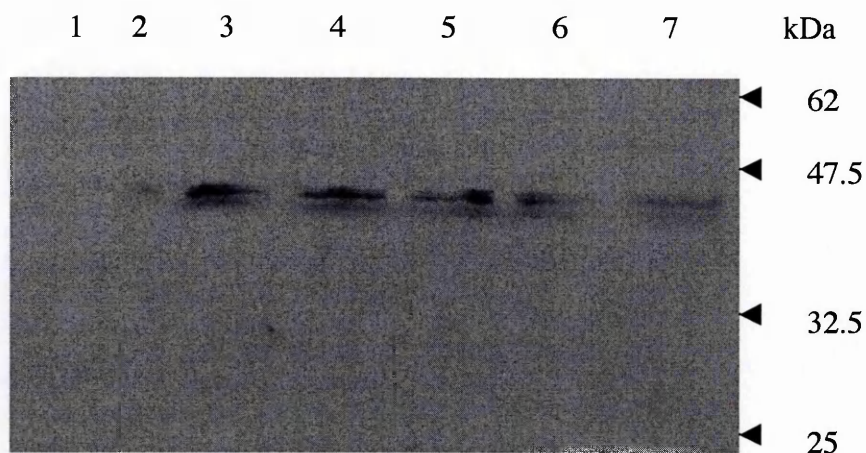


Figure 3.9 Detection of scFv-B2-CaM by immunoblot. A stable S2 cell line expressing B2-CaM was scaled up in 200 ml serum free medium before induction with 500  $\mu$ M  $\text{CuSO}_4$ . 1 ml samples were taken at days 1-7 post induction and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. CaM tagged proteins were detected with peroxidase labelled N9A and visualised by enhanced chemiluminescence. Lanes 1-7, days 1-7.

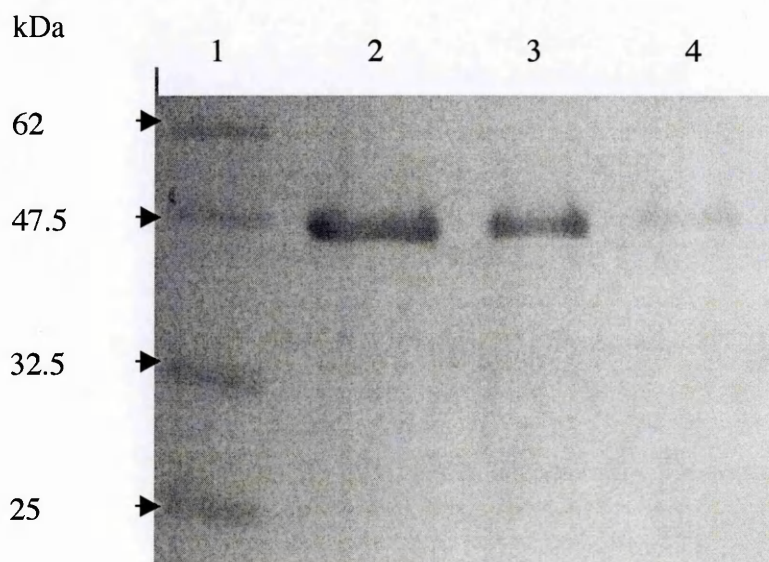


Figure 3.10 Coomassie stained SDS-PAGE gel of purified scFv-B2-CaM. S2 cells were treated as for Figure 3.5. On day 5 post induction 200 ml of supernatant was harvested and supplemented with 1 mM  $\text{CaCl}_2$ . scFv B2-CaM was purified over a W7 column and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu$ l of each fraction analysed on a 12% SDS-PAGE gel and visualised with Coomassie blue (Chpt 2.2.2.8.1). Lane 1- broad range molecular weight marker, Lanes 2-4 – fractions 2-4.

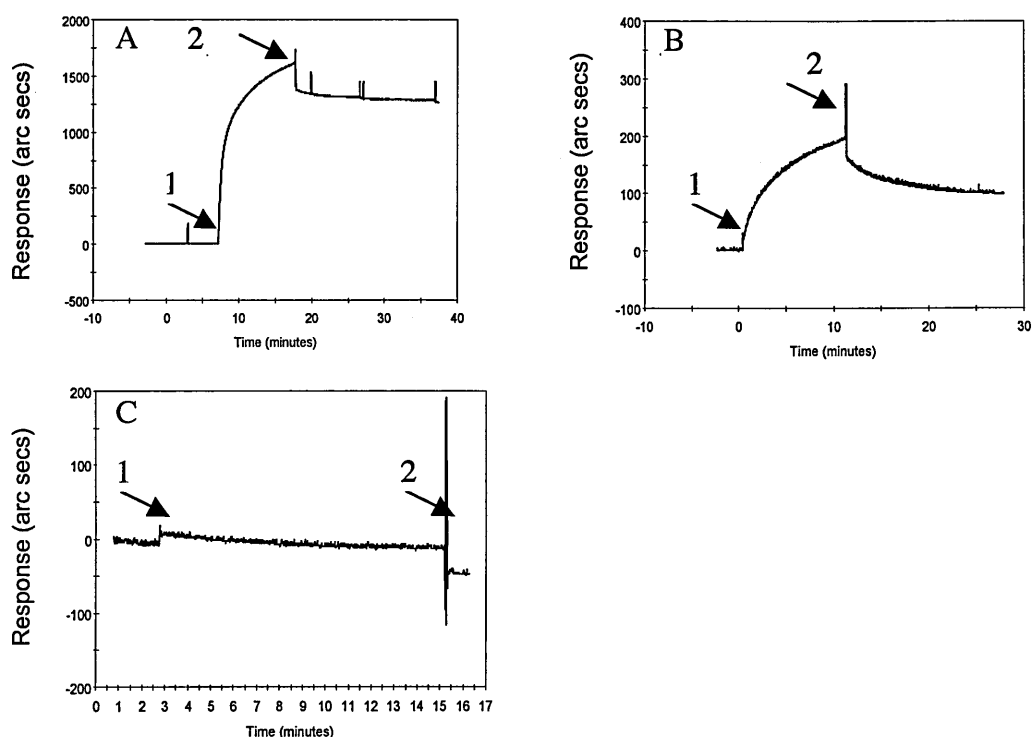


Figure 3.11 Binding of both HPA-1 alleloforms of GPIIb/IIIa to B2-CaM measured by resonant mirror technology. The arrows at position 1 indicate the addition of GPIIb/IIIa and the arrows at position 2 indicate the addition of wash buffer. A) Addition of recombinant B2-CaM to BSA-N9A in the presence of calcium, the baseline was re-set to zero after the removal of excess B2-CaM, B) Addition of purified GPIIb/IIIa-Leu 33 alleloform (HPA-1a). C) Addition of purified GPIIb/IIIa-Pro 33 alleloform (HPA-1b). Representative of n=2.



### **3.3.5 Expression of patient scFvs in *Drosophila* S2 cells**

Twenty-five patients with low-grade follicular lymphoma were enrolled in the phase I/II LIFTT clinical trial (CRC code: 98/04, protocol No: PH2/039). The V<sub>H</sub>/V<sub>L</sub> gene cassettes were assembled in pVAC2 by our collaborators in Southampton, Manchester and Bournemouth. In short, RNA was recovered from biopsy material and reverse transcribed to cDNA with isotype and kappa or lambda specific forward primers. The V<sub>H</sub> and V<sub>L</sub> genes were amplified with the appropriate sets of V gene and J gene primers. The amplified V<sub>H</sub> and V<sub>L</sub> genes were cloned and at least ten clones each for V<sub>H</sub> and V<sub>L</sub> were sequenced. The dominant sequence, assumed to be derived from the malignant clone, was selected for the construction of the V<sub>H</sub>/V<sub>L</sub> gene cassette. The nucleotide and amino acid sequences of all 25 are shown in Appendix A. The V gene cassettes from the first three patients (B02, M01 and S01) enrolled in the trial were removed from the pVAC2 plasmid by restriction enzyme digestion with NcoI and NotI and sub-cloned into the pMT/BiP/V5 His D and pMT/BiP/CaM expression plasmids. Confirmation of V gene insertion was by sequence analysis.

#### **3.3.5.1 Transient expression of patient scFvs with His and CaM tags**

The pMT plasmids containing patient B02, M01 and S01 (Table 2.1) V gene cassettes were transfected into S2 cells. The level of expressed scFvs in the supernatant 5 days post induction was monitored by Western blotting (Figures 3.12 A and B, Figure 3.13). There appeared to be no expression of the scFvs from pMT/BiP/V5 His D as detected by C-terminal anti-His (Figure 3.12 A), however the anti-V5 antibody

produces a clear band at approximately 32 kDa (Figure 3.12B) indicating that the scFv is expressed and secreted but the C-terminal His tag has been removed. scFv-S01 appears smaller than the other three, which is compatible with their calculated molecular weights (MW) (Appendix B), M01, B01 and B2 are approximately 1 kDa heavier than S01. Also the potential for glycosylation will have an affect on the apparent MW (Table 3.1), the presence of glycosylation for the V5/His tagged proteins was not tested. The expression of the three scFvs with a CaM tag was successful and appropriate MW bands (~42 kDa) are detected with N9A-HRP (Figure 3.13). Again scFv-S01 is smaller than the other two. Based on these results the decision was taken to express the scFvs from the remaining 22 patients enrolled in the study as CaM tagged scFvs only.

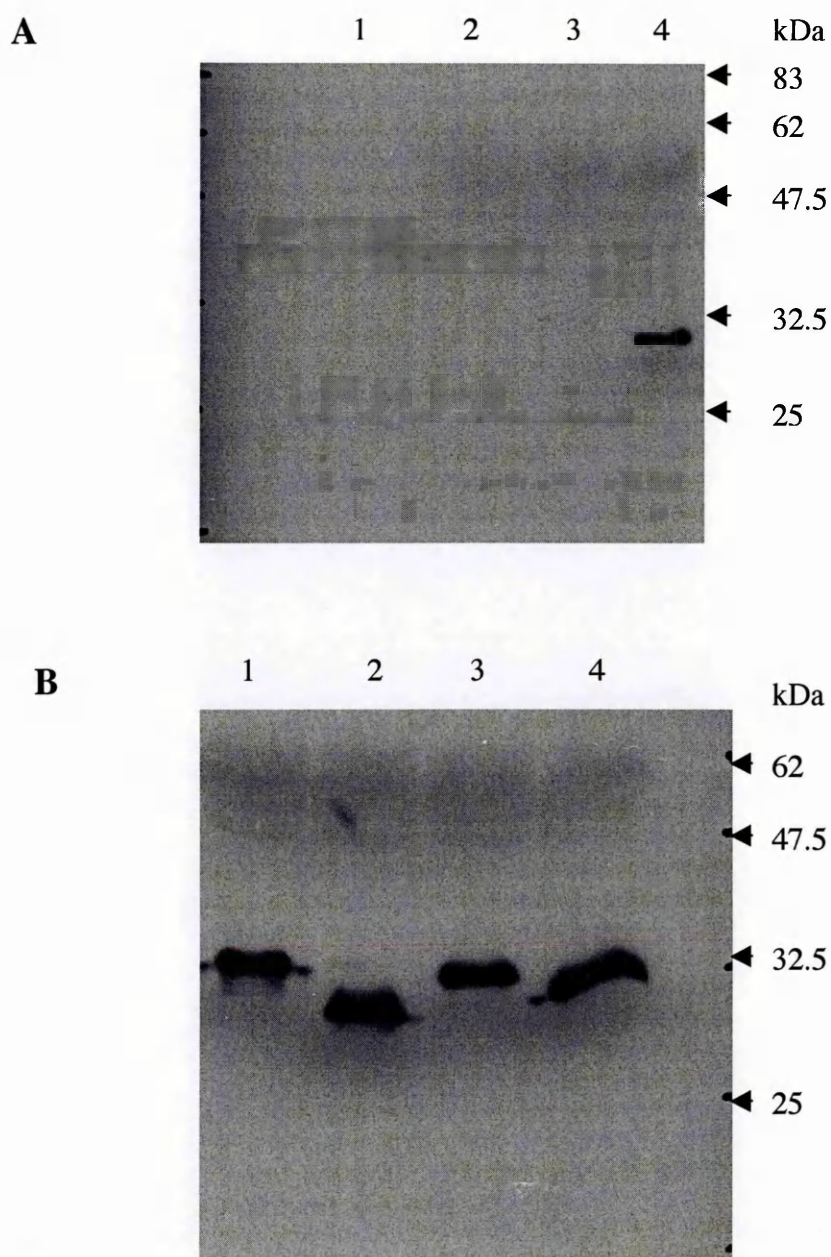


Figure 3.12 Detection of patient scFv-His by immunoblot. Transient S2 cell lines expressing V5/His tagged scFv-M01, S01, B02 and B2 were induced with 500  $\mu$ M  $\text{CuSO}_4$ . Supernatants were harvested on day 5 post induction and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. A) V5/His tagged scFvs were detected with peroxidase labelled mouse anti-His and visualised by enhanced chemiluminescence. B) V5/His tagged scFvs were detected with polyclonal anti-V5 and visualized as in A). Lanes 1-4, M01, S01, B02 and B2 respectively.

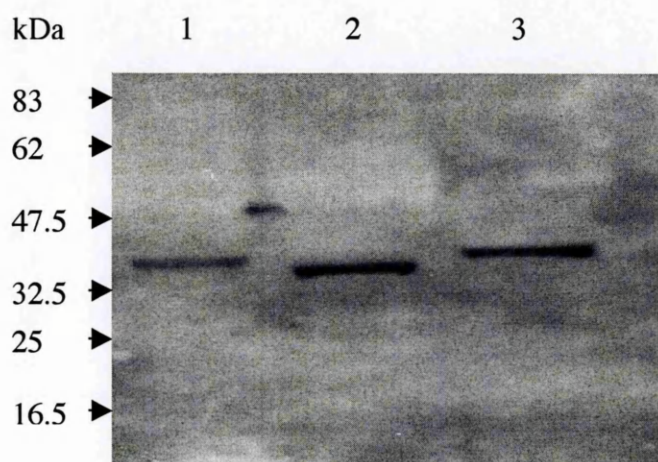


Figure 3.13 Detection of patient scFv-CaM by immunoblot. Stable S2 cell lines expressing scFv-CaM B02, M01 and S01 were treated as in Figure 3.7. On day 5 post induction a 1 ml sample was taken and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. CaM tagged scFvs were detected with peroxidase labelled N9A and visualised by enhanced chemiluminescence. Lanes 1-3 scFv-CaM M01, S01 and B01.

### 3.3.5.2 Stable expression of twenty-five patient scFvs with a CaM tag

The V<sub>H</sub>/V<sub>L</sub> gene cassettes of 25 FL patients (Table 2.1) were sub-cloned from pVAC2 into pMT/BiP/CaM; confirmation of correct gene insertion was by sequence analysis. The level of expressed scFvs in the supernatant 5 days post induction was determined by Western blotting (Figure 3.14). The expression levels of the scFvs in the supernatant appear to vary, for example M05 is barely detectable by this method (Figure 3.14 A).

The 25 stable lines were grown up in 200 ml of serum free medium and expression induced for 5 days. The supernatants were harvested on day 5 and purified over W7 columns; the eluted fractions were analyzed on a 12% SDS-PAGE gel and stained with Coomassie blue (Figure 3.15). The concentration of protein in the purified fractions was determined by BCA assay (Table 3.1).

Coomassie blue staining revealed the purified protein; the bands are shown in Figure 3.15 A-M. Purification of the scFv-CaM proteins should yield one band corresponding to the molecular weight of the scFv when analysed by SDS-PAGE gel and coomassie staining. However, analysis of the purified protein revealed several features. Firstly, as expected the molecular weight (MW) of the dominant band varied between clones. The MW of each scFv was calculated from the amino acid sequences, 10 of the 25 samples had a single band at the predicted MW. Secondly, for some clones there is an additional band at approximately 25 kDa (clones M01, S01, S05, B01 and B03). This fragment does not bind the N9A peptide as it was not revealed on the immunoblots in Figure 3.14. The nature of this band was investigated by N-terminal sequencing

(performed by the PNAC facility, Department of Biochemistry), which revealed that it was FK (Fujisawa Kaihatsu) binding protein 13 (FKBP13). FKBP13 is a family of proteins that bind the immuno-suppressive drug FK 506. FKBP13 (Genbank accession NP 476973) is encoded in the *Drosophila* genome (Bryant *et al.*, 1999). The amount of FKBP13 seems to be associated with cell viability. The expression level of FKBP13 is particularly high in the B03 clone (Figure 3.15 K) this culture only had 50% viable cells, as determined by trypan blue staining and FKBP13 accounts for approximately 50% of the total purified protein. A repeat culture and purification of B03 (Figure 3.16) where the cells were >98% viable at harvest shows a substantial reduction of the contaminating FKBP and conversely a substantial increase in the yield of scFv. Thirdly, for several clones two bands are observed in the expected MW range. This is most prominent for clones M04, M06, B04, S011, S013, S017 and S018. The doublet is most likely caused by glycosylation of the V domains. This observation is consistent with the presence of one or more putative N-linked glycosylation sites (Table 3.1) except for M04 and S017, which contain none. All purified scFvs were subsequently assayed for the presence of N-linked glycosylation. ELISAs were performed using HRP labelled Concanavalin A (Con A) (Chpt 2.2.2.4). Of the purified scFvs 11 are N-glycosylated (Figure 3.17) as detected by Con A-HRP. A sample was negative for glycosylation if the binding was less than 20% of the strongest Con A binder. Finally, the amount of scFv-CaM obtained varied widely between clones. This was calculated by measuring the concentration of protein in all preparations (Table 3.1).

There was no detectable protein from the 200 ml cultures of clones M05, M02 and S06 (data not shown). The results for M02 and S06 were surprising as there was expression present in the 3 ml transient cultures as detected by Western blotting (Figure 3.14 D).

The difference between the test cultures and the 200 ml cultures is that the latter is in shake-flasks and 'serum free' conditions. To investigate whether the failure of expression was associated with the absence of foetal calf serum (FCS) parallel test cultures were set up in complete *Drosophila* Expression System (DES) medium and serum free medium for clones M02 and S06. This confirmed our assumption that the loss of expression was associated with the withdrawal of FCS. To further verify this a 400 ml culture of clones M02 and S06 in shake-flasks was maintained for 5 days in complete medium with 10% FCS, supplemented with 0.1% Pluronic F-68 solution (Sigma). Supernatants were harvested and purified over W7 columns and the eluted fractions were analyzed on a 12% SDS-PAGE gel and stained with Coomassie blue (Figure 3.18 A and B). The absence of expression of scFv-CaM was associated with the lack of FCS for two of the three clones (M02 and S06). This could be resolved by growing the cells in the presence of FCS. An obvious disadvantage of this approach is that the purity of scFv-CaM after a single purification step is substantially lower and significant amounts of albumin is co-purified. Expression of M05 was never achieved.

| Patient number | Amount of protein (mg) | Number of N-X-S/T sites | Predicted N-glycosylation* | Detected N-glycosylation# |
|----------------|------------------------|-------------------------|----------------------------|---------------------------|
| B01            | 5.0                    | 2                       | 2                          | ✗                         |
| B02            | 6.0                    | 1                       | 0                          | ✗                         |
| B03            | 1.7                    | 2                       | 1                          | ✗                         |
| B04            | 2.2                    | 1                       | 1                          | ✓                         |
| B05            | 1.5                    | 1                       | 1                          | ✗                         |
| M01            | 3.0                    | 4                       | 3                          | ✗                         |
| M02            | 0.2                    | 1                       | 1                          | ✓                         |
| M03            | 3.2                    | 4                       | 4                          | ✗                         |
| M04            | 1.1                    | 0                       | 0                          | ✓                         |
| M05            | 0                      | 1                       | 0                          | nt                        |
| M06            | 0.3                    | 2                       | 2                          | ✓                         |
| M08            | 1.2                    | 2                       | 2                          | ✗                         |
| S01            | 3.4                    | 2                       | 2                          | ✗                         |
| S02            | 0.4                    | 1                       | 1                          | ✓                         |
| S03            | 0.3                    | 3                       | 3                          | ✓                         |
| S04            | 1.0                    | 2                       | 2                          | ✗                         |
| S05            | 2.3                    | 2                       | 1                          | ✗                         |
| S06            | 0.8                    | 1                       | 0                          | ✓                         |
| S07            | 2.2                    | 1                       | 1                          | ✗                         |
| S09            | 17.0                   | 5                       | 3                          | ✗                         |
| S011           | 2.0                    | 2                       | 1                          | ✓                         |
| S012           | 0.1                    | 4                       | 3                          | ✓                         |
| S013           | 1.4                    | 1                       | 1                          | ✗                         |
| S017           | 0.5                    | 0                       | 0                          | ✓                         |
| S018           | 0.5                    | 2                       | 1                          | ✓                         |
| B2             | 4.0                    | 0                       | 0                          | ✗                         |
| CaM            | nt                     | 1                       | 1                          | nt                        |
| B2-His         | 3.0                    | 0                       | 0                          | ✗                         |

Table 3.1 scFv protein yields as determined by bicinchoninic acid (BCA) assay. The concentration of the purified scFv in each of the fractions was determined by BCA assay, yields are expressed as the total amount of purified protein obtained from a 200 ml culture supernatant. \* as determined by NetNGlyc server (*R. Gupta, E. Jung and S. Brunak*. In preparation, 2004). # As detected by Con A ELISA. nt – not tested, ✓ - glycosylation detected, ✗ - no glycosylation detected.



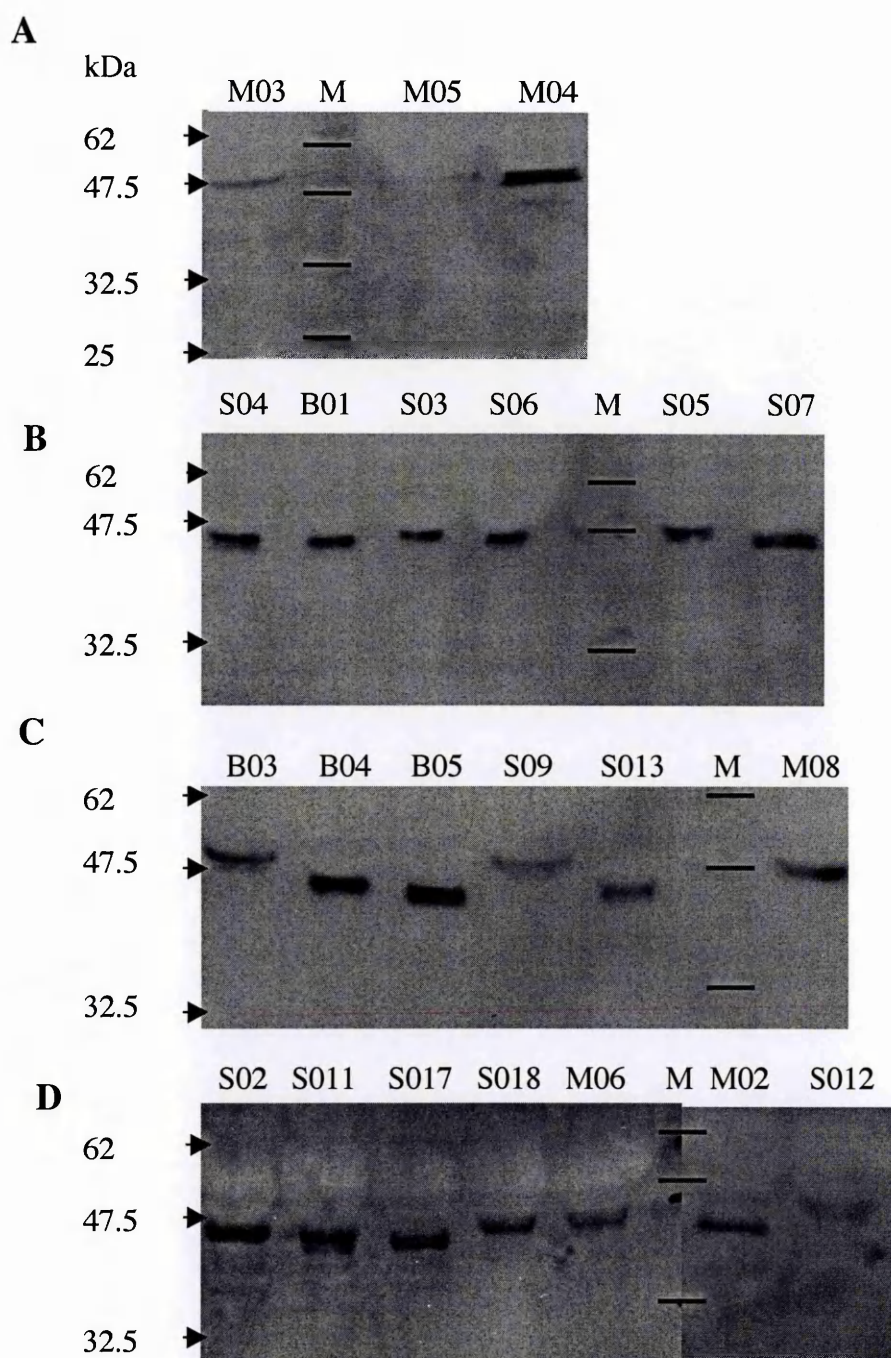
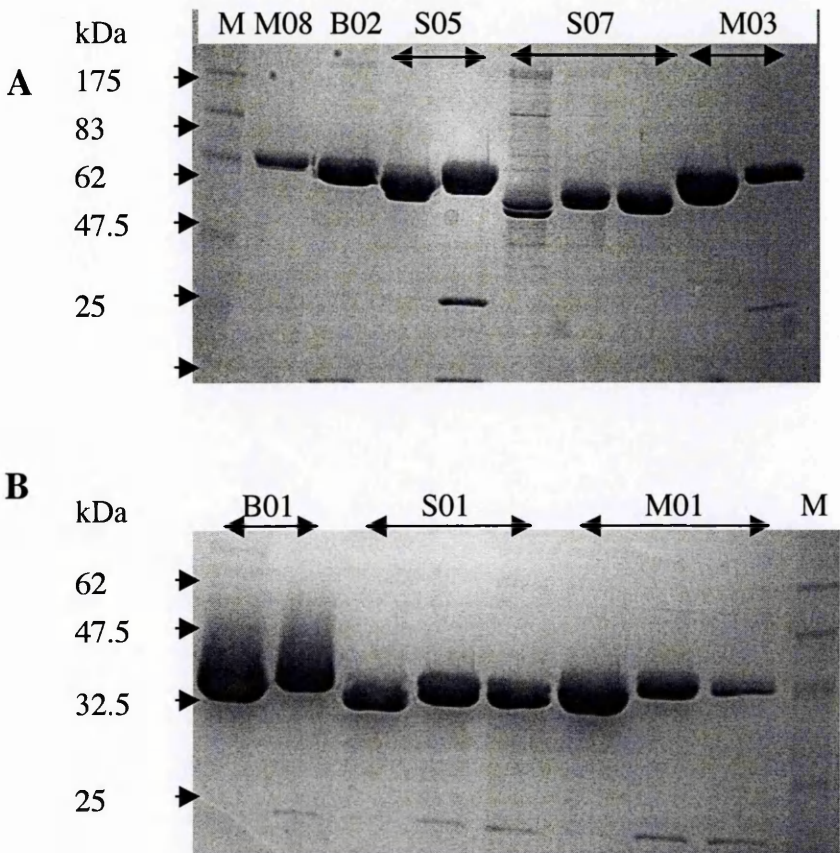


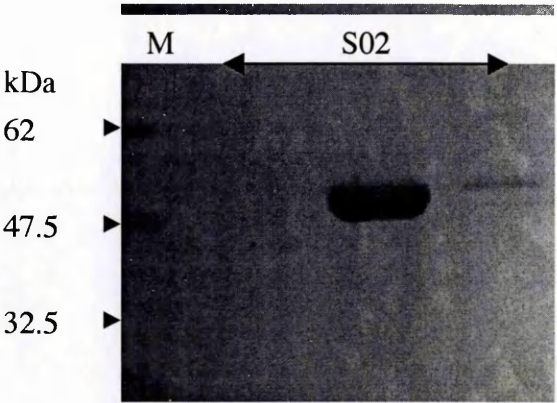
Figure 3.14 Detection of patient scFv-CaM by immunoblot. 22 Stable S2 cell lines expressing scFv-CaM fusion proteins were treated as in Figure 3.9. On day 5 post induction 1 ml sample was taken and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. CaM tagged proteins were detected with peroxidase labelled N9A and visualised by enhanced chemiluminescence. The patient identifiers are presented above the corresponding lane. Molecular weight markers (M) are drawn on the blots and the sizes are indicated by arrows.

Figure 3.15 A-M Coomassie stained SDS-PAGE gels of purified scFv-CaM.

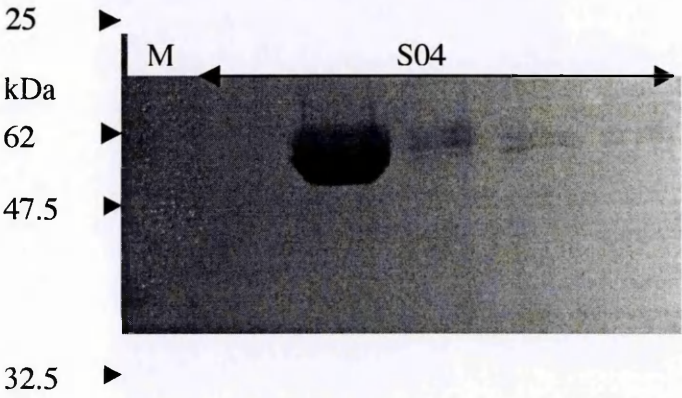
S2 cells were treated as for Figure 3.5. On day 5 post induction 200 ml of supernatant was harvested and supplemented with 1 mM  $\text{CaCl}_2$ . scFvs were purified over W7 columns and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu\text{l}$  of each fraction containing protein as detected by BCA assay were analysed on a 12% SDS-PAGE gel and stained using Coomassie blue.



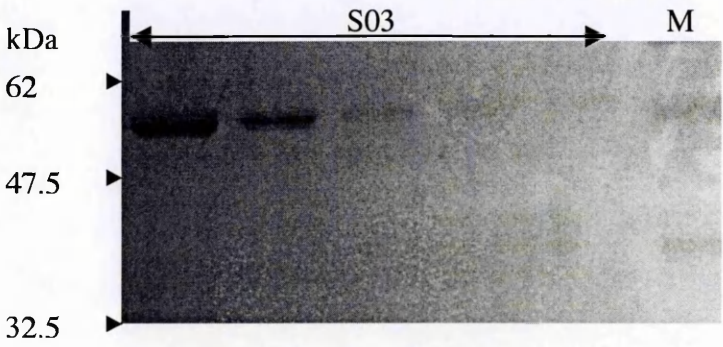
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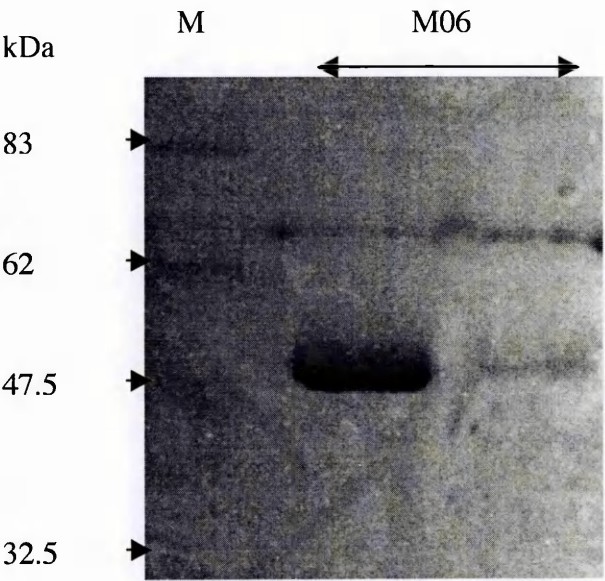


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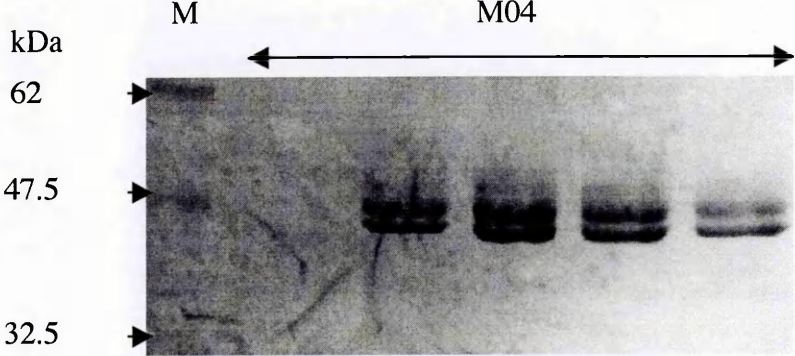




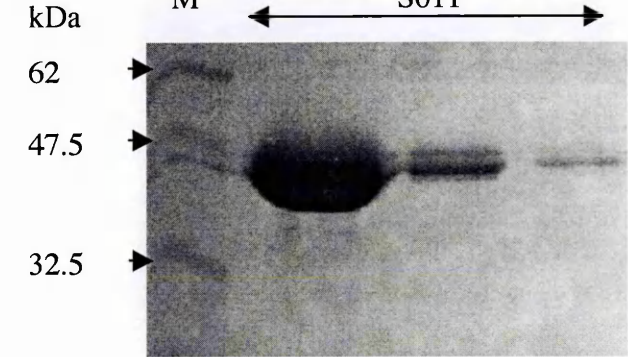
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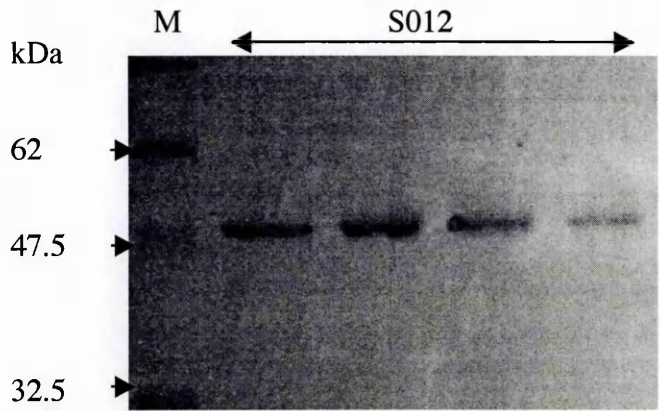
**G**



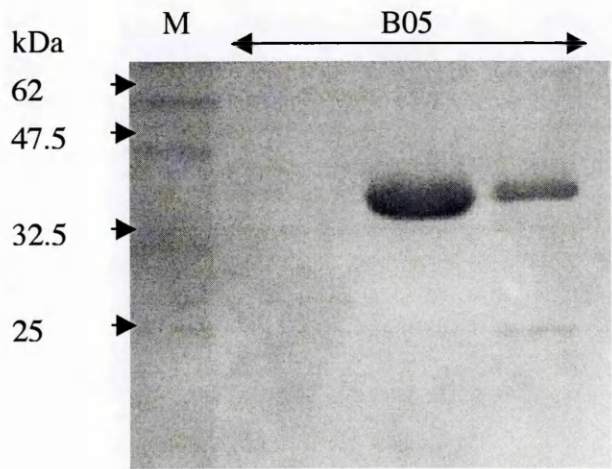
**H**



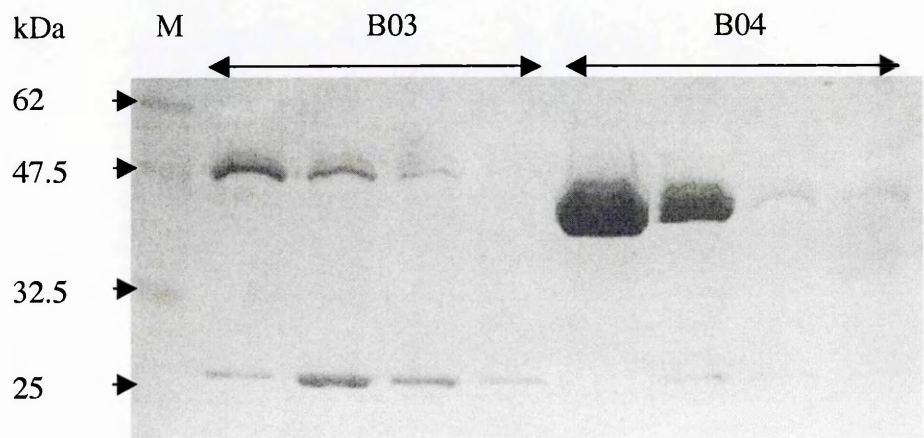
**I**



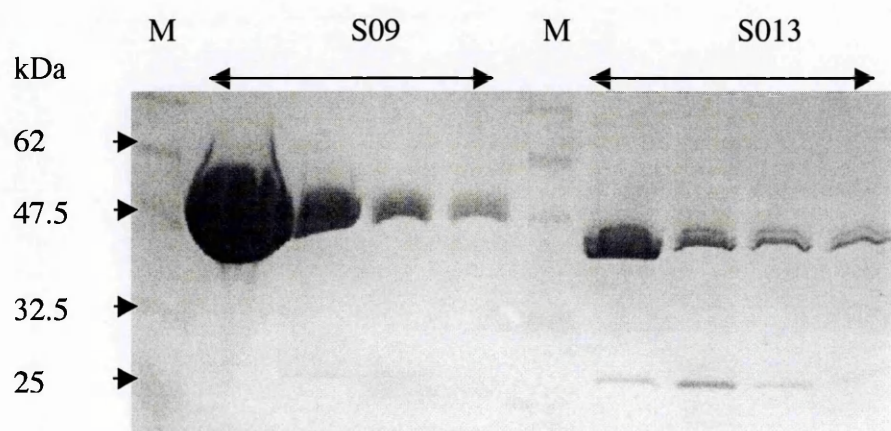
**J**



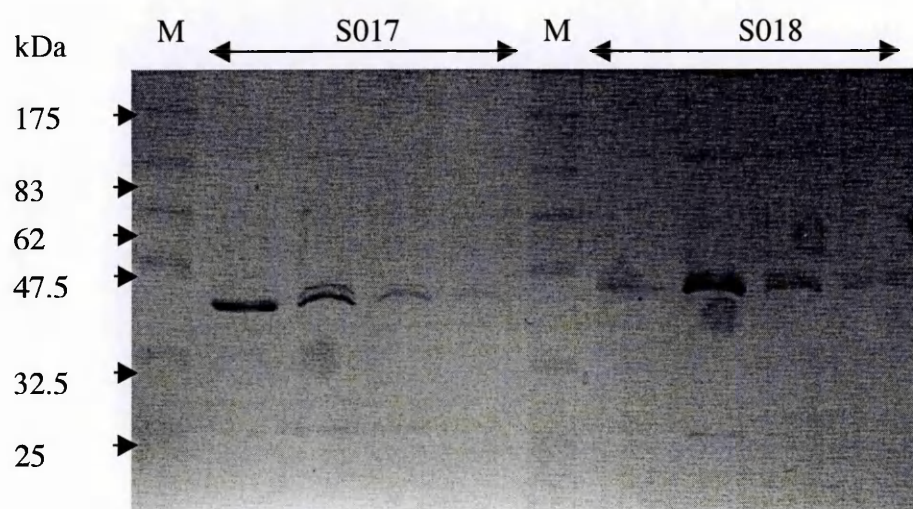
**K**



**L**



**M**



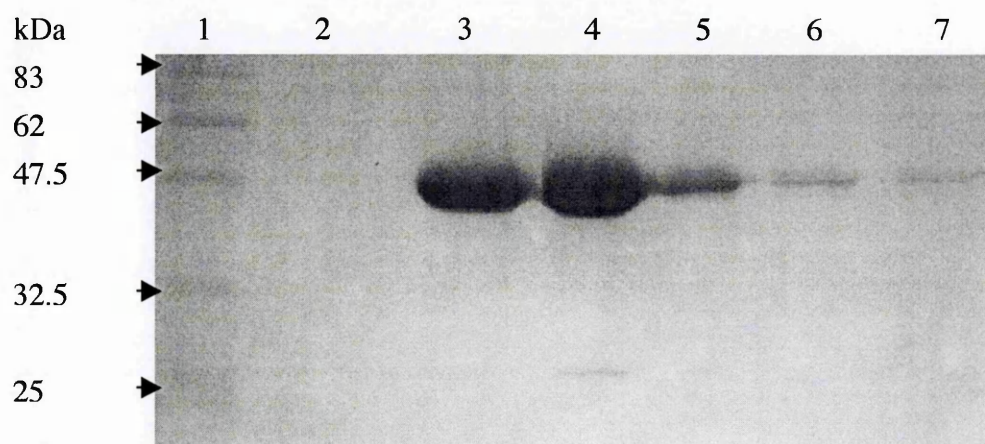


Figure 3.16 Coomassie stained SDS-PAGE gel of purified B03-CaM. S2 cells were treated as for Figure 3.5. On day 5-post induction 200 ml of supernatant was harvested and supplemented with 1mM  $\text{CaCl}_2$ . scFv B03-CaM was purified over a W7 column and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu\text{l}$  of each sample containing protein as detected by BCA assay were analysed on a 12% SDS-PAGE gel and stained using Coomassie blue. Lane 1 - broad range molecular weight marker, Lanes 2-7 – fractions 1-6.

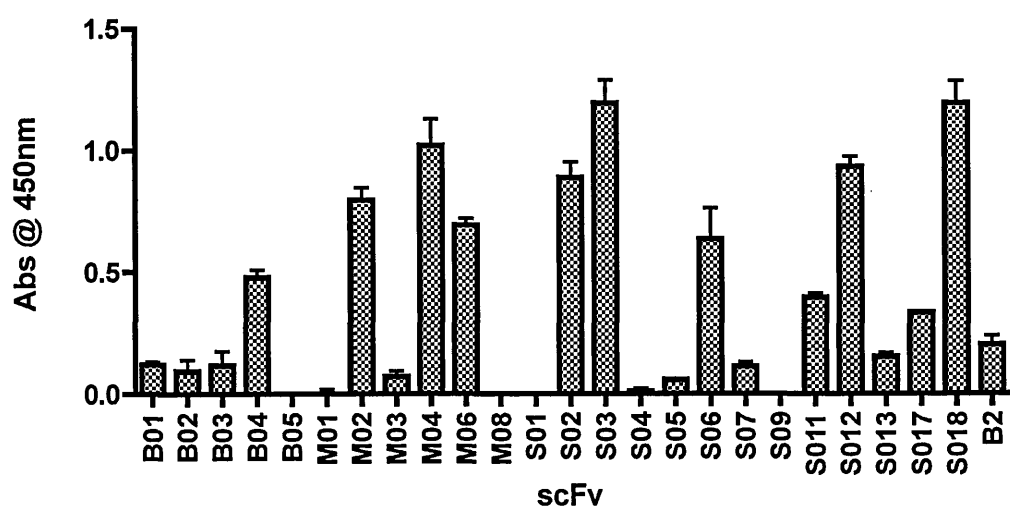


Figure 3.17 Binding of Con A-HRP to purified scFv-CaM. BSA-N9A was coated onto ELISA plates and CaM tagged scFvs were subsequently captured. Detection of N-linked glycosylation was by binding of Con A-HRP to each scFv.



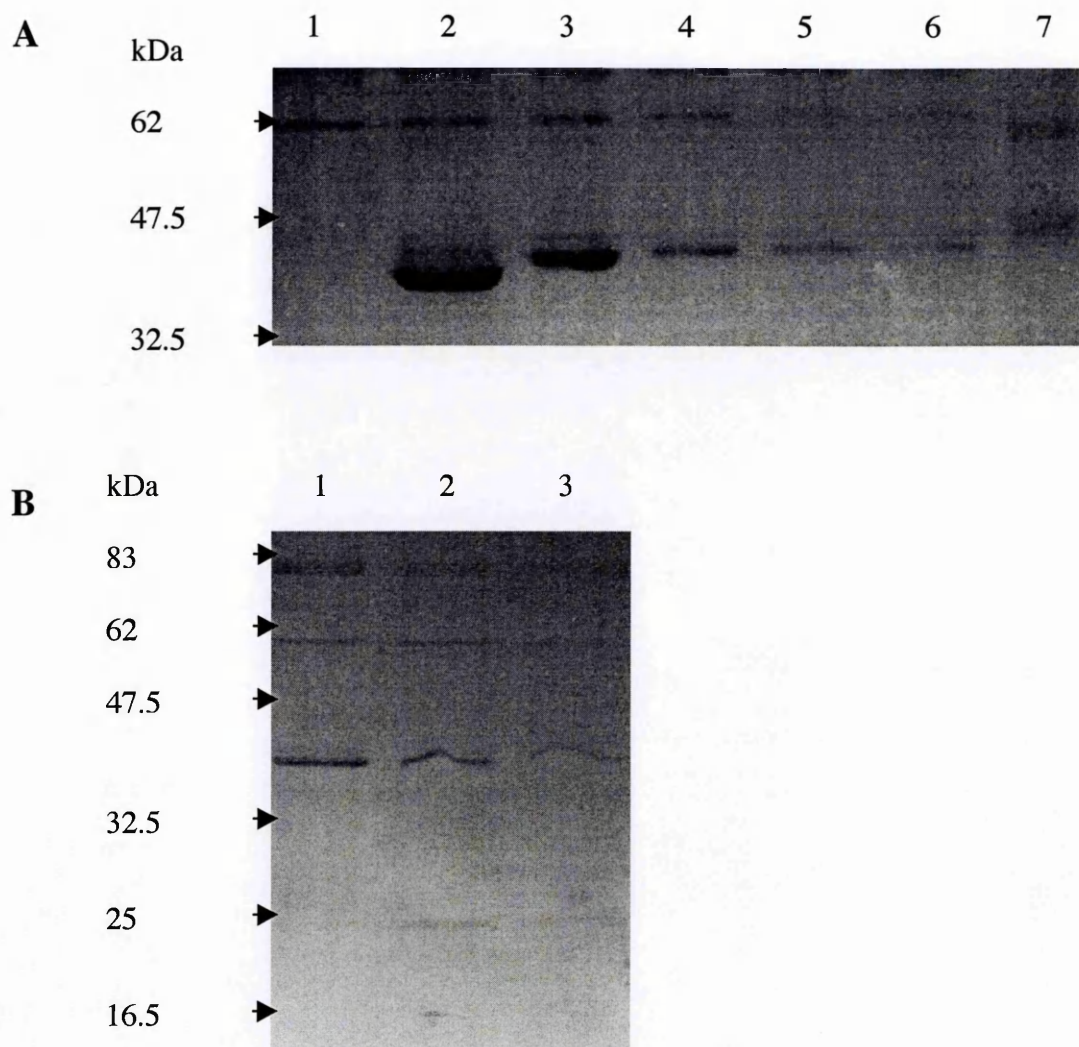


Figure 3.18 Coomassie stained SDS-PAGE gels of S06-CaM and M02-CaM. Stable S2 cell lines expressing S06 and M02-CaM were scaled up into 400 ml of complete DES media before induction with 500  $\mu$ M  $\text{CuSO}_4$ . On day 5 post induction 400 ml of supernatant was harvested and supplemented with 1mM  $\text{CaCl}_2$ . scFv-CaM was purified over a W7 column and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu$ l of each sample containing protein as detected by BCA assay were analysed on a 12% SDS-PAGE gel and stained using Coomassie blue. A) S06-CaM Lane 7 - broad range molecular weight marker, Lanes 1-6 – fractions 1–6, B) M02-CaM Lanes 1-3 – fractions 2-4.

### 3.4 Discussion

The work described in this chapter concerns the construction and subsequent analysis of two expression vectors for the expression of scFvs in *Drosophila* S2 cells. The ultimate aim of this work was to provide a simple, reliable strategy for the expression and purification of a large panel of scFvs. A well-characterised scFv specific for the leu-33 alleloform of platelet GPIIbIIIa, from the laboratory, scFv-B2 was initially used to assess the efficacy of both plasmids, particularly the performance of the purification tags (His and CaM) and the functional activity of the final purified scFv. A comparison was also made between the *Drosophila* expressed scFv-B2 and scFv-B2 derived from a TG1 *E. coli* expression system that had previously provided adequate scFv, in both quality and quantity (Griffin & Ouwehand, 1995).

The expression data for scFv-B2 shows that the *Drosophila* expression system is capable of producing between 15 mg/L (B2-V5/His) and 20 mg/L (B2-CaM) compared to 1 mg/L from the TG1 *E. coli* expression system (B2-myc-His). The overall yield is apparently increased by the use of the CaM tag. There are two possible explanations for this. Firstly, purification of the CaM tagged protein does not require any dialysis pre or post purification. Secondly, the propensity for cleavage of the His tag means that not all of the protein produced may be available for purification using the nickel-agarose columns. Moreover, the scFv-CaM fusion appears less prone to cleavage. Evidence shown in this chapter for scFv-B2 also shows that the *Drosophila* derived protein has a better functional activity than its *E. coli* counterpart as established by the IC50 value in Figure 3.8. Approximately 2-fold more of the *E. coli* produced scFv was needed to achieve 50% inhibition of binding of the polyclonal anti-idiotypic antibodies. This may be explained by the quality of the protein, eukaryotic

systems tend to produce better folded and therefore more active protein than prokaryotic systems (reviewed in Verma et al (Verma *et al.*, 1998)). Interestingly both scFvs were unable to produce 100% inhibition of binding whilst IgG1 B2 at 80 µg/ml did result in complete inhibition. There could be several reasons for the incomplete inhibition of the binding of the polyclonal IgG by the recombinant proteins. A potential explanation is that the polyclonal serum contains a population of antibodies reactive to the constant domains of IgG1 B2 that are not present in the scFvs. However, this is unlikely as the rabbits were only immunised with a vaccine devoid of constant domains. Alternatively bivalency is required to produce complete inhibition and a population of the polyclonal antibodies does bind with a far greater avidity to B2 IgG1 than to scFv-B2. Finally the highest concentration of scFv was too low, but this seems unlikely, as a plateau had been reached at 74% and 80% for *E. coli* and *Drosophila* respectively with a concentration of scFv at 20 µg/ml.

The functional activity of B2-His was also assessed by the platelet immunofluorescence test (PIFT). B2-His bound to GPIIbIIIa on HPA-1a1a positive but not -1b1b platelets. This method could not be used to test B2-CaM because the binding of the N9A peptide to CaM is calcium dependant and platelets become prone to aggregation in the presence of calcium. Therefore, real-time binding of purified GPIIbIIIa to B2-CaM was shown using mirror resonance technology. The CaM binding peptide N9A, conjugated to BSA, was immobilized to the surface of the cuvette; the B2-CaM was added in the presence of calcium and subsequently captured. The purified GPIIbIIIa from HPA-1a1a platelets bound to the recombinant scFv whereas GPIIbIIIa from -1b1b platelets did not. Both recombinant scFvs B2-His and

B2-CaM have allelic specificity with no binding to the proline-33 alleleform of GPIIbIIIa but robust binding to the leucine-33 alleleform.

Expression in *Drosophila* using the pMT/BiP/V5 His D plasmid did not provide the same degree of success for the first three patient derived scFvs. Although the scFv was expressed in each case as detected by the binding of anti-V5, the anti-His antibody failed to bind. The most likely explanation for this is that partial cleavage of the C-terminal end of the fusion protein had occurred, removing some or all of the histidines preventing binding of the anti-His. Without the histidine tag the fusion protein could not be purified using a simple nickel-chelating column. However, the same three scFvs were then successfully expressed using pMT/BiP/CaM, which incorporates the gene for calmodulin in place of the V5 and His tags in pMT/BiP/V5-His D and purified using a W7 column. In total 25 out of 26 scFvs were expressed and purified using the *Drosophila* CaM tag system. Only one scFv, M05, failed to yield any purified protein. Analysis of the amino acid sequence for codon usage, potential post-translational modifications and the isoelectric point (pI) have failed to offer any explanations for the lack of expression. The post purification yields of scFvs produced from the *Drosophila* system varies from 0.5 mg to 85 mg/L, the majority produce at least 2.5 mg/L. In comparison expression of scFv in *E. coli* can result in yields of 5-7 mg/L in shake flask cultures this requires careful optimisation of the growth conditions and is limited to a small number of scFvs (Verma *et al.*, 1998; Ziegler & Torrance, 2002). The development of an expression system for the production of recombinant scFv with C-terminal Calmodulin tags in *Drosophila* S2 cells permits simple, single-step affinity purification of tagged antibody from culture supernatants without the need for cell

lysis or dialysis. The final fusion product remains stable in the EDTA elution buffer at 4 °C for several months (data not shown) with no evidence of proteolytic cleavage.

The CaM tag can also be utilized to capture / immobilize the recombinant scFv onto a solid surface, for example in ELISA or mirror resonance technology (this chapter and Li et al 2000) (Li *et al.*, 2000). The 23-mer alanine mutant (N9A) derived from myosin light-chain kinase (Montigiani *et al.*, 1996) is bound directly to the surface and the fusion protein is directly captured by means of the CaM tag, the scFv of interest is therefore likely to be maintained in its native configuration with the epitopes available for binding of the anti-idiotypes than if it had been immobilized directly. This immobilisation has been successfully used in mirror resonance technology (Chpt 2.2.5 and figure 3.11) and in ELISA, which will be discussed in chapter 4. The  $K_d$  between CaM and N9A is in the order of  $10^{-12}$  allowing direct capture of the fusion protein from supernatants thus facilitating a simple single step purification.

The apparent differences in the molecular weights of the expressed scFvs, as visualised by Coomassie blue staining cannot be explained solely by the calculated molecular weights of each scFv (Appendix B). V genes derived from the malignancies of patients with Follicular Lymphoma have been shown to contain more putative N-linked glycosylation sites than is usually expected (Zhu *et al.*, 2002). In this chapter it has been shown that the *Drosophila* expression system is capable of N-linked glycosylation as demonstrated by the ability of some of the scFvs to bind Con A (Figure 3.17). There are several observations that can be made from the binding of Con A to the scFvs. Firstly, as expected in eight clones, binding of the Con A to scFvs that contain predicted glycosylation sites was observed and conversely in three clones,

no binding of Con A to scFvs with no predicted sites. Secondly, in 11 clones with predicted sites no binding of Con A was observed. Although the Asn-X-Ser/Thr (N-X-S/T) sequon is generally required for N-linked glycosylation, many such sequons are glycosylated inefficiently (Plummer & Hirs, 1964; Pohl *et al.*, 1984; Thim *et al.*, 1988; Curling *et al.*, 1990; Shakin-Eshleman *et al.*, 1992) or not at all (Gavel & von Heijne, 1990). Finally, binding of Con A to three scFvs that contain no predicted sites was observed in M04, S06 and S017. S06 does contain one N-X-S/T sequon although it was not predicted to be glycosylated. In rare cases other sequence motifs have been shown to support glycosylation (Gavel & von Heijne, 1990), although these are not present in either M04 or S017. However, both scFvs do contain several asparagine residues, which the *Drosophila* cells may be able to glycosylate. In the majority of cases the variability in the molecular weights of the purified proteins can be explained by a combination of the primary amino acid sequence and by the presence or absence of N-linked glycosylation. For M08, B03 and S09 that have been shown not to contain N-linked glycosylation, the increased molecular weight could be due to the presence of O-linked glycosylation, which *Drosophila* is capable of achieving.

The viability of the S2 cells plays an important role in the quality of the purified recombinant scFv. Loss of viability at the time of harvest reduces the yield of purified scFv for two reasons. Firstly, cell death allows the release of proteases from the cytoplasm, which causes proteolysis, and secondly FKBP is also released and competes for binding to W7 during purification.

Overall, although expression in *E. coli* is effective for the production of scFv antibody fragments and high yields can be achieved it is not the ideal system for the

simultaneous production of large amounts of many proteins. The *Drosophila* system with the CaM tag, though initially more time consuming due to the production of stable lines, has several clear advantages over the *E. coli* and baculovirus systems described by Neri and Li (Neri *et al.*, 1995b; Li *et al.*, 2000). Firstly, several cell lines can be maintained at the same time and protein expression can be induced when required. Secondly, manipulation of several samples can be done simultaneously as smaller volumes of culture supernatant are needed to provide milligram yields. Thirdly, it produces high yields of good quality scFv. Finally once the stable cultures have been established they can be maintained at room temperature, without the need for specialised incubators.

The pMT/BiP/V5 His D and pMT/BiP/CaM have been successfully used by collaborating scientists to express a number of scFvs (personal communication Nancy Wandersee, The Blood Centre of Southeastern Wisconsin, USA) and other proteins containing immunoglobulin folds, for example human glycoprotein VI (GPVI) (Smethurst *et al.*, 2004) and the triggering receptor expressed on myeloid cells (TREM) like transcript-1 (TLT-1) (Barrow *et al.*, 2004). An original manuscript detailing the use of calmodulin tagged D1D2 (extracellular domains of GPVI) for phage antibody isolation has been accepted for publication in Journal of Immunological Methods (Jennings *et al.*, 2006).

The *Drosophila* expression system and the C-terminal Calmodulin tag provide an excellent protein expression system, with high yields and purity.

## Chapter 4A

### **Production of clinical grade DNA vaccines for administration to Follicular Lymphoma patients enrolled in the Cancer Research UK clinical trial (PH2/039)**

#### **4.1 Introduction**

##### **4.1.1 Follicular Lymphoma**

Follicular Lymphoma (FL) is one of over 20 different types of Non-Hodgkin's Lymphoma (NHL); NHL can be split into B cell and T cell lymphomas. FL is a B cell lymphoma and makes up about 25% of all NHL. FL can occur at anytime during adulthood, with the average age of onset in the sixth decade. Painless swelling in the neck, armpit or groin, due to enlarged lymph nodes, some loss of appetite and tiredness are the most common symptoms. A definitive diagnosis is performed by lymph node biopsy and microscopy, giving a histological classification. Histology further classifies the B cell NHL into low-grade, intermediate-grade and high-grade varieties ([www.cancerbacup.org.uk](http://www.cancerbacup.org.uk))(Harris *et al.*, 1994). Although these varieties of tumours are usually indolent and only slowly progressive, they seldom respond to conventional forms of treatment (chemotherapy and radiotherapy). Despite the fact that most follicular lymphomas are advanced at the time of diagnosis, the median survival of patients with follicular lymphomas is approximately 10 years, and allogeneic bone marrow/stem cell transplantation can induce long-term remissions in these patients (Forrest *et al.*, 2002), but transplant-associated mortality is high.



Autologous bone marrow transplantation has low transplant-associated mortality, but most patients relapse after this modality of therapy. More recently trials with humanized antibodies against CD20 as adjuvant therapy seem to substantially improve the outlook for FL patients (Maloney *et al.*, 1994; Maloney *et al.*, 1997a; Maloney *et al.*, 1997b; Davis *et al.*, 1999). Recent studies have shown that the analysis of the transcriptome of FL cells does allow better categorization of disease genotype and the transcriptome signatures provide a better predictor for disease severity and survival than the morphological grading and clinical prognostic parameters currently used (Alizadeh *et al.*, 2000; Glas *et al.*, 2005).

#### **4.1.2 The tumour specific target**

For several reasons FL has become a model disease for immunotherapy. Firstly, impressive results were achieved in a small human trial infusing monoclonal anti-idiotypic in nine patients (Kwak *et al.*, 1992). Complete tumour regression was reported in two patients. Secondly, the waxing and waning properties typical of lymphomas suggests a role for immune surveillance in FL. Finally, FL may result from profound immunosuppression, for example post-transplant lymphoproliferative disorders (PTLD). The incidence of PTLD has increased as a direct result of the long term use of cyclosporin A (CyA) and the new very potent immunosuppressants such as anti-CD3 monoclonal OKT3 and FK506 given for the prevention of transplant rejection (Penn *et al.*, 1969; Otto *et al.*, 1987; Boubenider *et al.*, 1997; Bates *et al.*, 2003). Many research groups using different immunological approaches have pursued the notion that the immune system could be exploited to take control of minimal residual disease (MRD) in FL.

The tumour cells express on their surface the B-cell differentiation markers, MHC class I and II molecules and monoclonal immunoglobulin (Ig) (Anderson *et al.*, 1984). The monoclonal Ig is unique to a given lymphoma clone, distinguishing it from the patients healthy B cells and the lymphomas of other patients (Stevenson & Stevenson, 1975; Levy *et al.*, 1977). Immunoglobulins consist of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulphide bonds (Roitt *et al.*, 2001) (Figure 1.3). The light chain consists of one constant and one variable (V) domain, whereas in the case of IgG the heavy chain consists of one variable and three constant domains. The antigen-binding site is composed by the pairing of two individual V domains, one from the heavy chain ( $V_H$ ) and one from the light chain ( $V_L$ ). The variable domains are made up of framework regions (FR1-4) and three short hypervariable loops of amino acids, the complementarity-determining regions (CDR1-3). The binding specificity of the surface Ig is determined by sequence variability in these loops, length variability of both CDR3's coupled with minor alterations in residues in the underlying  $\beta$ -sheet framework, which serve to adjust the orientation of individual loops.

During B cell development, random assembly of  $V_H$ , D (Diversity) and J (Joining) gene segments on the heavy chain locus of a V and J gene on either of the light chain loci results in the de novo formation of a coding  $V_H$  or  $V_L$  gene respectively. A process of somatic hypermutation can then alter the sequence of the rearranged V genes, which is an important mechanism for affinity maturation (Neuberger & Milstein, 1995).

Therefore, each B cell expresses a unique combination of  $V_H$  and  $V_L$  domains and hence a unique antigen binding site. The antigen-binding site itself is also an antigenic determinant and the collection of epitopes that it produces is known as the idiotype (Slater *et al.*, 1955). It can therefore be the target of an anti-idiotypic response.

The clonally derived population of transformed B cells in NHL expresses surface Ig whose idiotype represents a truly unique tumour-specific antigen (Stevenson & Stevenson, 1975). It is believed that immunotherapeutic strategies directed against the lymphoma idiotype will be able to mediate anti-idiotypic and / or cytotoxic (CTL) responses to elicit tumour destruction.

A phase I trial of idiotypic protein vaccination in 41 patients with NHL has been reported (Hsu *et al.*, 1997). Briefly, heterohybridomas were prepared from tumour biopsies by fusion; clones expressing tumour derived Ig were expanded and idiotypic Ig were purified for administration to patients. The outcome of this study was suggestive that idiotypic vaccination is a potentially beneficial approach in the treatment of FL (Hsu *et al.*, 1997). However the time and labour involved in making each patient specific vaccine, typically several months, limits the wider clinical application of this approach. Recent advances in antibody engineering and the advent of vaccination with naked plasmid DNA mean that patient-specific idiotypic DNA vaccines can now be readily produced. Pre-clinical studies in mice immunised with scFv DNA vaccine produced good cytotoxic and humoral responses against idiotype (Stevenson *et al.*, 1995; Spellerberg *et al.*, 1997; King *et al.*, 1998). The immunisation against the idiotype by means of DNA vaccination is simpler and DNA vaccines for a phase I and phase I/II DNA vaccination trial in FL have been prepared in our laboratory (Hawkins *et al.*, 1997; Stevenson *et al.*, 2004).

#### **4.1.3 pVAC2**

The plasmid (pVAC2) used in this trial contains the V<sub>H</sub>/V<sub>L</sub> gene cassette derived from the patient's malignant clone fused to the gene for fragment C of tetanus toxoid (FrC)

used as an immune enhancing element (Spellerberg *et al.*, 1997). Tetanus Toxoid (TT) is a 1315 amino acid protein produced by the bacterium *Clostridium tetani* and is the most powerful neurotoxin known to man (Kisasato, 1889). It is composed of a heavy and light chain joined by disulphide bonds. FrC is the 451 amino acid, 50 kDa carboxy terminal fragment of the heavy chain. It is a highly immunogenic protein with several MHC class II restricted T cell epitopes (Demotz *et al.*, 1989) and also has a promiscuous universal T helper cell epitope (TT 947-967), which can be presented by multiple Human Leucocyte Antigen (HLA) class II and murine MHC alleles (Panina-Bordignon *et al.*, 1989). FrC has been used as an adjuvant in licensed clinical vaccines such as Hib (Plotkin & Orenstein, 1988) and in several experimental vaccines against *Plasmodium falciparum* and *Plasmodium berghei* (Valmori *et al.*, 1992). A plasmid DNA vaccine encoding FrC has been shown to produce potent antibody responses and provide protection against TT challenge in mice (Anderson *et al.*, 1996). It has also been shown that anti-idiotypic responses in DNA vaccinated mice can be enhanced up to 50 times with FrC (Spellerberg *et al.*, 1997) and the mean survival time after challenge with A31, a B-cell splenic lymphoma cell line is also increased (King *et al.*, 1998).

#### **4.1.4 Assembly of $V_H$ / $V_L$ gene cassette**

Rapid DNA sequencing can now identify the idiotypic immunoglobulin from RNA isolated from a tumour biopsy in a matter of days. The rearranged antibody V genes are amplified individually using the polymerase chain reaction (PCR), these amplified DNA fragments are then cloned and sequenced, and the tumour V genes, predominant in the starting material, are identifiable as recurring sequences. The tumour cell V

genes can then be assembled as a single chain antibody fragment (scFv), in which a short glycine-serine peptide linker (GGGGS)<sub>3</sub> connects the V<sub>H</sub> and V<sub>L</sub> domains (Huston *et al.*, 1991).

#### 4.1.5 Clinical Trial

The outcome of the phase I clinical trial (PH1/027) did not show signs of obvious toxicity when administering escalating doses of naked plasmid DNA in patients with advanced stage FL (Hawkins *et al.*, 1997). The objectives of the phase I/II trial, Study of Idiotypic Vaccination for Follicle Centre Lymphoma (LIFTT) (PH2/039), discussed here were to establish whether patients with FL can generate an immune response against, 1) a known immunogen (FrC) and 2) a tumour derived antigen (scFv), delivered by a DNA vaccine. The eligibility criteria for patient entry into the trial are given in Table 4.1.

The plasmid containing the scFv was directly administered into the deltoid muscle by simple needle injection, whereupon it is taken up and expressed by cells near the injection site (Wolff *et al.*, 1990; Jiao *et al.*, 1992; Wolff *et al.*, 1992). The expressed protein is processed by antigen presenting cells (APCs) to elicit both humoral and cellular immune responses.

| Inclusion   | Exclusion   |
|---|---|
| Histologically confirmed low-grade FL expressing surface Ig         | Chemo or radiotherapy in the 10 weeks preceding proposed commencement of immunisation       |
| Clinical complete remission   | A monoclonal band on serum electrophoresis  |
| Life expectancy > 1 year  | Pre-existing anti-DNA, anti-muscle, rheumatoid factor activity or active autoimmune disease |
| Minimum age 18 years  | Positive for Human Immunodeficiency Virus (HIV) or Hepatitis B Virus (HBV)                  |
| Lymph node biopsy prior to chemotherapy suitable for RNA extraction | Other malignancies  |
| Platelet count > 50 x 10 <sup>9</sup> / L                           | Pregnant or lactating   |
| Signed informed consent   | Receiving other anti-cancer therapy   |
|   | Withdrawal of consent   |

Table 4.1 Eligibility for patients in the LIFTT (PH2/039) clinical trial.

Once accepted the patient was given a unique study identifier and RNA was extracted from a lymph node biopsy. Twenty-five FL patients were enrolled in the trial (Table 4.2), which was designed as a dose escalation study with 5 patients receiving 6 immunisations of the designated dose over a 12-week period; the doses of DNA vaccine ranged from 0.5 mg – 2.5 mg. Each dose group must have completed their course of immunisations before patients could be enrolled at the next dose level. The duration of the trial from first to last vaccination was 50 months (from November 1999 to January 2004).

#### **4.1.6 Testing the DNA vaccines in rabbits**

The validated patient vaccines were administered to Dutch rabbits; serum was assayed pre and post DNA vaccination for anti-tetanus and anti-idiotypic responses. The recombinant scFv-CaM proteins (Chapter 3) were used in ELISA to detect the anti-idiotypic responses in these rabbits. The anti-tetanus and anti-idiotypic data provided positive controls for the efficacy of the plasmid vaccine and a control for the scFvs and anti-idiotypic assay. Once the assays were validated using rabbit serum the serum samples from a selected number of patients were also tested for the presence of anti-tetanus and anti-idiotypic IgG pre and post immunisation.

| Dose Level | Patient Identifier        | Dose Received (mg) |
|------------|---------------------------|--------------------|
| 1          | B02, M01, M02, S01, S02   | 0.5                |
| 2          | B01, M03, M04, S03, S04   | 1.0                |
| 3          | M05, M06, S06, S07, S011  | 1.5                |
| 4          | B03, B04, S09, S012, S013 | 2.0                |
| 5          | B05, M08, S05, S017, S018 | 2.5                |

Table 4.2 Unique patient identifier for the LIFTT (PH2/039) clinical trial. The dose of pVAC2-scFV each patient received is shown. The letter denotes the registration centre of the patient, B - Royal Bournemouth Hospital, Bournemouth, M - Christie Hospital, Manchester and S - Southampton General Hospital, Southampton.



## **4.2 Experimental Design**

All reagents purchased for the making of the DNA vaccines were British Pharmaceutical (BP) grade or similar where possible. The lot numbers and date of receipt were recorded. The water used for making media or performing dilutions of the reagents was sterile water for irrigation and was purchased from Addenbrookes pharmacy. Any reagents used were made and sterilised on site and assigned unique batch numbers and 12-month expiration dates.

The vaccines were produced under Good Manufacturing Procedures (GMP) and subjected to pre-release sterility and quality checks after they had been aliquoted into sterile vials for administration. Each vaccine was assigned a unique batch number.

### **4.2.1 Cloning of scFv in pVAC2**

The patient  $V_H/V_L$  gene cassettes were assembled in pVAC2 at the trial centres in Southampton and Manchester. The pVAC2-scFv DNA along with a sequence file and alignments to the closest germline  $V_H$  and  $V_L$  genes were transferred to Cambridge. In our laboratory the cassettes were transformed into batch recorded DH5 $\alpha$  competent cells (Gibco) (Chpt 2.2.1.9) and a single colony was grown O/N in LB media (Chpt 2.2) and used to inoculate 5 L of LB media in a 7 L fermentor (Applikon).

### **4.2.2 Giga prep of DNA vaccine**

Plasmid DNA was isolated using Qiagen Gigaprep (Chpt 2.2.1.12), the contents of the buffers are listed in Appendix C.

The fermented DH5 $\alpha$  containing pVAC2-scFv DNA was pumped from the fermentation vessel into five sterile centrifuge bottles and centrifuged at 18000g for 20 min at 4°C. Each pellet was resuspended in 125 ml of buffer P1, the bacteria was then lysed by the addition of 125 ml of buffer P2, mixed by inversion and incubated at RT for 5 min. The lysed bacteria was then neutralised with 125 ml of chilled buffer P3, mixed by inversion and each added to a QIAfilter Mega-Giga cartridge and incubated at RT for 10 min. The liquid was pulled through the filter using a vacuum. The filter was then washed with 50 ml of FWB2 and pulled through using a vacuum; the precipitate was stirred gently to aid filtration. The filtered lysates were transferred to a class II safety cabinet and a 75  $\mu$ l sample was taken from each for an analytical gel to determine whether growth and lysis conditions were optimal (sample 1 a-e). The filtered lysates were then applied to pre-equilibrated Qiagen-tip 10000s and allowed to enter the resin by gravity flow. A 75  $\mu$ l sample of each flow-through was taken for an analytical gel to determine the efficiency of DNA binding to the Qiagen resin (sample 2 a-e). Each Qiagen-tip was then washed with a total of 600 ml of buffer QC. A 120  $\mu$ l sample of the combined wash fractions was taken for an analytical gel (sample 3 a-e). The DNA was then eluted from the Qiagen-tips with 100 ml of buffer QF. A 22  $\mu$ l sample of each eluate was taken for an analytical gel (sample 4 a-e). The DNA was precipitated by the addition of 70 ml of isopropanol and centrifuged at 18000 g for 30 min at 4°C. The supernatant was removed and each pellet resuspended in 10 ml of 70 % ethanol and centrifuged as above for 10 min. The pellets were air-dried for 10 min and resuspended in a total volume of 10 ml of sodium chloride injection BP 0.9% w/v (Phoenix Pharma Ltd). The DNA pellet was then subjected to two rounds of ethanol precipitation (Chpt 2.2.1.13) then resuspended in sodium chloride as above to the appropriate concentration. A 2  $\mu$ l sample of the final product was taken for the

analytical gel (sample 5). Samples 1-5 were analysed on a 1.2% agarose gel (Chpt 2.2.1.3).

#### **4.2.3 Sterile Fill of DNA vaccine**

The DNA vaccines were aliquoted into nitrogen filled sterile vials (Amersham) in a Grade A laminar flow cabinet in a Grade B aseptic suite designed for sterile manipulation of blood, blood products and tissues at the National Blood Service Cambridge. All equipment entering the room was autoclaved in double bags where appropriate and sprayed with isopropanol. Personnel entering the room were required to wear single use all-in-one suits, including surgical facemasks and hats. The outermost covers of the equipment were removed before placing in the laminar flow cabinet. Once the vials had been filled they were removed from the cabinet and labelled before being sealed in individual bags. Only one patient vaccine was allowed in the room at any one time.

#### **4.2.4 Vaccine validation**

##### **4.2.4.1 Sterility of DNA vaccines**

The sterility test was performed in the Public Health Laboratory (PHLS East), Addenbrookes Hospital, Cambridge. Two sterile filled vials of DNA vaccine were added under sterile conditions into two bottles of BacT/Alert® FAN® aerobic culture bottles and incubated at 37°C for 5 days. Any growth was recorded and an Environmental Microbiology Report was filed.

#### **4.2.4.2 Endotoxin Levels in the DNA vaccines**

The amount of endotoxin in each vaccine was tested at two laboratories, the Bio Products Laboratory (BPL), Hertfordshire and National Institute for Biological Standards and Controls (NIBSC), Hertfordshire. The endotoxin levels were measured in Endotoxin Units (EU) or International Units (IU) /ml, which is equivalent to 0.1 ng endotoxin/ml. For the vaccine to be accepted the endotoxin level was required to be below 2500 EU/ml. The vaccine was accepted based on the highest level recorded.

#### **4.2.4.3 Quality checks of the DNA vaccine**

After the sterile fill each DNA vaccine was sequenced (Chpt 2.2.1.14) and the sequence compared to the original that was provided at the transfer of the pVAC2-scFv cassette to Cambridge. The vaccine plasmids were also digested with Sall, HindIII / NotI and SfiI / NotI (Chpt 2.2.1.15) and the sizes analyzed on a 1.2% agarose gel (Chpt 2.2.1.3).

#### **4.2.5 Immunisation and assay of sera**

To test the efficacy of each of the patient vaccines, one Dutch rabbit was immunised i.m. with each of the patient vaccines (Chpt 2.2.3.1). Each rabbit was initially vaccinated with 200 µg of pVAC2-scFv plasmid DNA in PBS injected at equal dose into two sites in the quadriceps muscles. Immunisations were performed at days 0, 14 and 28. Bleeds were taken at days 0 and 35 to measure antibody responses. Serum was

assayed by ELISA for the presence of IgG against tetanus toxoid (Chpt 2.2.4.1), cognate idiotype and non-cognate idiotype for cross reactivity (Chpt 2.2.4.2).

#### **4.2.6 Site directed mutagenesis and expression of mutants**

scFvs B04 and B05 each contain one putative N-linked glycosylation site at residues 50 and 99 respectively. These Putative glycosylation sites are identified by the following amino acid sequons, B04 Asn-Ile-Thr and B05 Asn-Ile-Ser. Primers were designed to mutate the asparagines to alanine residues (Table 4.3) and used in Stratagene XL site directed mutagenesis kit (Chpt 2.2.1.17).

| Primer<br>Code | Sequence 5' to 3'                         | Melting<br>point<br>(T <sub>m</sub> °C) |
|----------------|---|---|
| B04<br>N50A F  | GCGACTGGAGTGGATTTTCAGCCATTACTAGTAGTGGC    | 78.3                                    |
| B04<br>N50A R  | GCCACTACTAGTAATGGCTGAAATCCACTCCAGTCGC     | 78.3                                    |
| B05<br>N99A F  | GTATTACTGTGCGAAGGCTATTAGTATGGAAATATGGGGCC | 78.2                                    |
| B05<br>N99A R  | GGCCCCATATTTCCATACTAATAGCCTTCGCACAGTAATAC | 78.2                                    |

Table 4.3 Nucleotide sequences and melting temperatures of the site directed mutagenesis primers. The nucleotide substitutions to mutate Asparagine to Alanine are shown in red. F (Forward primer) is the sequence of the sense strand, R (Reverse primer) is the sequence of the antisense strand.

#### 4.2.7 Clinical Trial patient serum

Patients received 6 doses of their tailor-made DNA vaccine over a 12-week period. Immunisations were given on weeks 0, 1, 2, 4, 8 and 12. Blood samples were taken on weeks 0, 1, 2, 3, 4, 8, 12, 16 and then every 4 weeks up to 52 weeks after the first immunisation. Patients unable to attend clinic at these specific time points were sampled at their closest outpatient appointment.

The work described in this chapter shows firstly the processes required to generate the clinical grade pVAC2-scFv DNA and the necessary sterility, endotoxin and plasmid structure checks needed before the vaccine can be administered. Secondly it reports on studies to establish whether our DNA vaccination plasmid pVAC2-scFv is capable of eliciting anti-tetanus and anti-idiotypic responses in Dutch rabbits. Thirdly, to what extent is the polyclonal anti-idiotypic response cross-reactive? Fourthly, do the patients in the clinical trial produce anti-idiotypic responses and are they showing an increase in anti-tetanus post vaccination? Finally, does the binding of anti-idiotypic IgG depend on the presence of the glycan moieties in the second and third CDRs of the V<sub>H</sub> domains?

### 4.3 Results

A summary of the 25 patient vaccines that were made and administered during the course of the trial is in Table 4.5. The vaccines that I prepared and checked are indicated by \*\*. The results shown in this chapter are from one of these vaccines (B05) and it is representative of all the vaccines prepared. Copies of the DNA vaccine preparation sheets completed in Cambridge are shown in Appendix E.

#### 4.3.1 Analysis of Giga-Prep

The aliquots taken during the DNA preparation (Chpt 4.2.2) were analysed on a 1.2% agarose gel (Figure 4.1). As expected the cleared lysate contains supercoiled and open circular plasmid DNA, lanes 1-5 (sample 1). Four of the five flow-through fractions, lanes 6-10 (sample 2) contain no DNA, indicating that the DNA binding to the resin was efficient. In lane 9 some of the DNA has passed through the column, compared to lane 4 this is a small fraction of the DNA initially added to the column. No bands were visible in lanes 11-15, no DNA was present in the combined wash fractions (sample 3), and the pure plasmid DNA remained on the column. Lanes 16-20 shows the eluates containing pure plasmid DNA. Interestingly it appears that the DNA was not equally distributed over the five columns, lanes 1-5 and 16-20. Lane 21 shows the aliquot of the pooled plasmid DNA from columns a-e.

The DNA from lane 21 was diluted 1:100 and the OD at 260 and OD 280 nm was measured (Table 4.4 A). The concentration was calculated using the equation in Chapter 2.2.1.2 and the 260:280 ratio was also recorded (Table 4.4 B) A ratio between 1.7 and 2.0 was accepted.



Patient B05 received the 2.5 mg dose; no further dilution of the DNA was needed and 16 vials were filled in total.

A)

| Wavelength (nm) | Optical Density (OD) |
|-----------------|----------------------|
| 260             | 0.491                |
| 280             | 0.269                |

B)

| Dilution Factor<br>(df) | Concentration (µg/ml)<br>OD @ 260nm x 50 x df | 260:280 ratio |
|-------------------------|---|---------------|
| 1:100                   | 2455  | 1.825         |

Table 4.4 DNA concentration and purity measurements. A) Optical density of B05 DNA vaccine at 260 and 280nm. B) Concentration and purity calculation of B05 DNA vaccine.

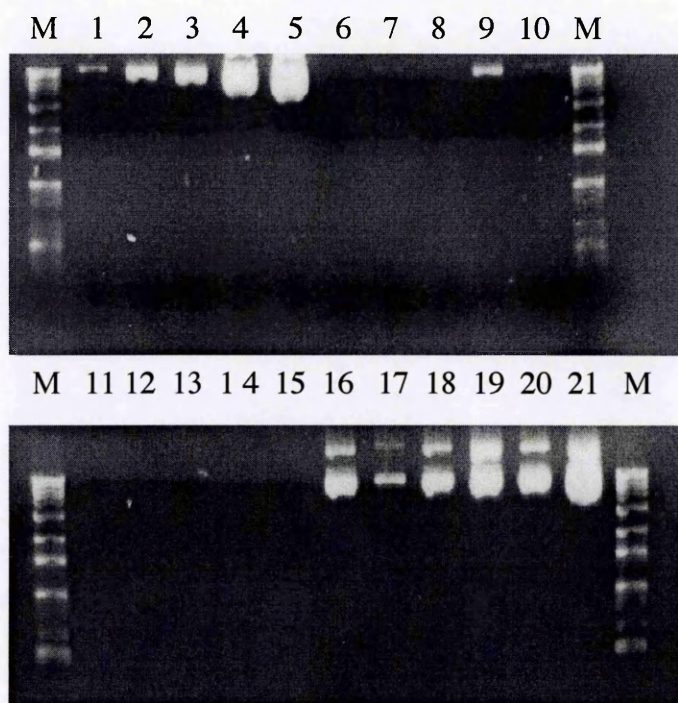


Figure 4.1 Agarose gel of samples recovered during Giga-prep plasmid DNA purification. Samples were removed and the DNA precipitated with 1 volume of isopropanol. The pellet was then washed with 200  $\mu$ l of 70% ethanol, the pellet was resuspended in 10  $\mu$ l of water, 2  $\mu$ l of each fraction was analysed on a 1.2% agarose gel. Five columns were used in the preparation of this vaccine; samples were taken from each column and labelled a-e. Lanes 1-5 cleared lysate a-e, Lanes 6-10 column flow-through a-e, Lanes 11-15 pooled wash a-e, Lanes 16-20 eluted DNA a-e, Lane 21 pooled, ethanol precipitated DNA vaccine, M – 1 Kb DNA marker.

## **4.3.2 Vaccine validation**

### **4.3.2.1 Sterility**

Two vials of vaccine were incubated in two vials of BacT/Alert® FAN® aerobic culture bottles and grown at 37°C for 5 days. No growth was reported (Figure 4.2) during this period, the vaccine is classed as sterile.

### **4.3.2.2 Endotoxin level**

The sterile vaccine was sent to BPL and NIBSC for endotoxin testing. The test results are shown in Figure 4.3 and 4.4 respectively. BPL measured the amount of endotoxin present as 14.4 EU/ml (Endotoxin Units) whereas NIBSC reported levels of between 3-6 IU/ml (International Units). 1 EU or IU/ml is approximately equal to 0.1 ng/ml. Both measurements fall within the maximum criteria so the vaccine is acceptable.

### **4.3.2.3 Plasmid structure**

The plasmid structure of the final product was confirmed by restriction enzyme digest analysed on a 1.5% agarose gel (Figure 4.5). Firstly, a SalI digest was performed. pVAC2 SalI digest should produce bands at 2745, 2188, 938, 63 and 34 base pairs (bp), whereas pVAC2-scFv SalI digest should produce bands at 2745, 2188, 1700 (approx.) and 34 bp. Figure 4.5 A confirms the predicted pattern of bands. The 68 and

34 bp fragments are not visible on the gel. Secondly, HindIII / NotI and SfiI / NotI double digests were performed on pVAC2-scFv. The HindIII / NotI digest should produce bands at approximately 900 and 4552 bp and the SfiI / NotI digest should produce bands at approximately 850 and 4606 bp. Figure 4.5 B confirms the predicted pattern of bands. pVAC2-scFv incubated with restriction enzyme buffer only provided an undigested control. Two bands are present, the upper band is supercoiled plasmid DNA and the lower band is nicked circular plasmid DNA (Figure 4.5 A and B). DNA sequencing confirmed that the sequence of the final product is identical to the sequence of the original pVAC2-scFv construct received from Bournemouth.

Cumulative record reference: A9384370 ZZDNAVACCINELAB

(Location: MEDNAVL )

Lab No: 02:ME0002596R BC#: 709380

Collected: 07/08/02 1115

Received: 07/08/02 1115

DNA VACCINE IN FAN BOTTLE X2

Source/place of sampling: WILKS, PAMELA  
Reason for request: Informal test

**REPORT:**

Sterility check Final  
No growth.

14/08/02-1551

**Figure 4.2 Environmental microbiology report for DNA vaccine B05. Sterile filled DNA vaccine was added to BacT/Alert® FAN® aerobic culture bottle and incubated at 37°C for a minimum of 5 days. No growth was recorded by 14/08/02, seven days after starting the incubation.**

LAL TEST FOR ENDOTOXINS

THIS IS RED IF BPL

LAL08

File Reference: LAL00805

CONTROLLED COPY

Written By: TJ Smith

LAL Test for Endotoxins

Authorised By: [Signature]

Date Effective: 8th August 2000

Sample: DNA VACCINE

Method of Testing: ~~Get~~ Clot/Turbidimetric\*

Sample: pH nk (indicate N/A if not applicable)

Sample Date: nk

| Sample Description      | Time Taken | Taken By | EU/ml |
|-------------------------|------------|----------|-------|
| PAMELA WILKS (FP02-201) | /          | /        | 14.4  |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |
|                         |            |          |       |

Tested By: Fanny/Ab

Date: 08 Aug 02

Tested according to SOP QBS 043 (16)

Result Checked By: [Signature]

Date: 14 AUG 2002

Signed: [Signature]

Date: 14 AUG 2002

Microbiology

\* delete as applicable

Figure 4.3 LAL (Lymulus amoebocyte lysate) test for endotoxin performed by Bio Products Laboratory (BPL) Hertfordshire. The results are given as Endotoxin units (EU) /ml, 1 EU/ml  $\approx$  0.1 ng/ml.

## LAL REQUEST FORM

|   |   |
|---|---|
| (1)   | (2)   |
| LAL sample No: <u>477/02</u>  | Manufacturer: <u>EABC</u>                   |
| Date booked in: <u>7.08.02</u>                                      | Lot/Batch No: <u>Pamela WILKS 805</u>       |
|   | Date results required: <u>asap</u>          |
| (3)   |   |
| Product Name: <u>Pamela WILKS DNA vaccine</u>                       | Requested by: <u>Paul M/Willem Overbeek</u> |
| Reconstitution Volume: <u>N/A</u>                                   | Division: <u>Haem</u>                       |
| Diluent Lot No: <u>—</u>  | SLA: <u>HA/4/87</u>                         |
| Maximum allowable endotoxin concentration (MAEC) in IU/ml: <u>—</u> |   |

SAMPLE (delete as appropriate)

This sample contains material of human origin: Yes/~~No~~ *DNA sequence only*

This is a new product or a new formulation of an existing product: ~~Yes~~/No

Sample pH is between 6 and 8: Yes/~~No~~

This product or its diluent contains aluminium hydroxide: ~~Yes~~/No

Dilutions of the sample may be prepared in plastic receptacles: Yes/~~No~~

Product to be collected after testing: Yes/~~No~~

|                |                       |                     |  |  |
|----------------|-----------------------|---------------------|--|--|
| (4)            |                       |                     |  |  |
| Test number    | : <u>02/126</u>       | <u>02/128</u>       |  |  |
| Date of test   | : <u>9.08.02</u>      | <u>9.08.02</u>      |  |  |
| Operator       | : <u>G. Tarrant</u>   | <u>G. Tarrant</u>   |  |  |
| Test method    | : <u>manual</u>       | <u>manual</u>       |  |  |
| Lysate         | : <u>Pyrotell Acc</u> | <u>Pyrotell Acc</u> |  |  |
| Result (IU/ml) | : <u>3-30</u>         | <u>3-6</u>          |  |  |

Measured endotoxin concentration : 3-6 IU/ml

Comments : \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Product Passes/Fails : N/AApproved : Pa me Date: 12.08.02

Figure 4.4 LAL test for endotoxin performed by National Institute for Biological Standards and Controls (NIBSC), Hertfordshire. The results are given as International units (IU) /ml, 1 IU/ml  $\equiv$  0.1 ng/ml.



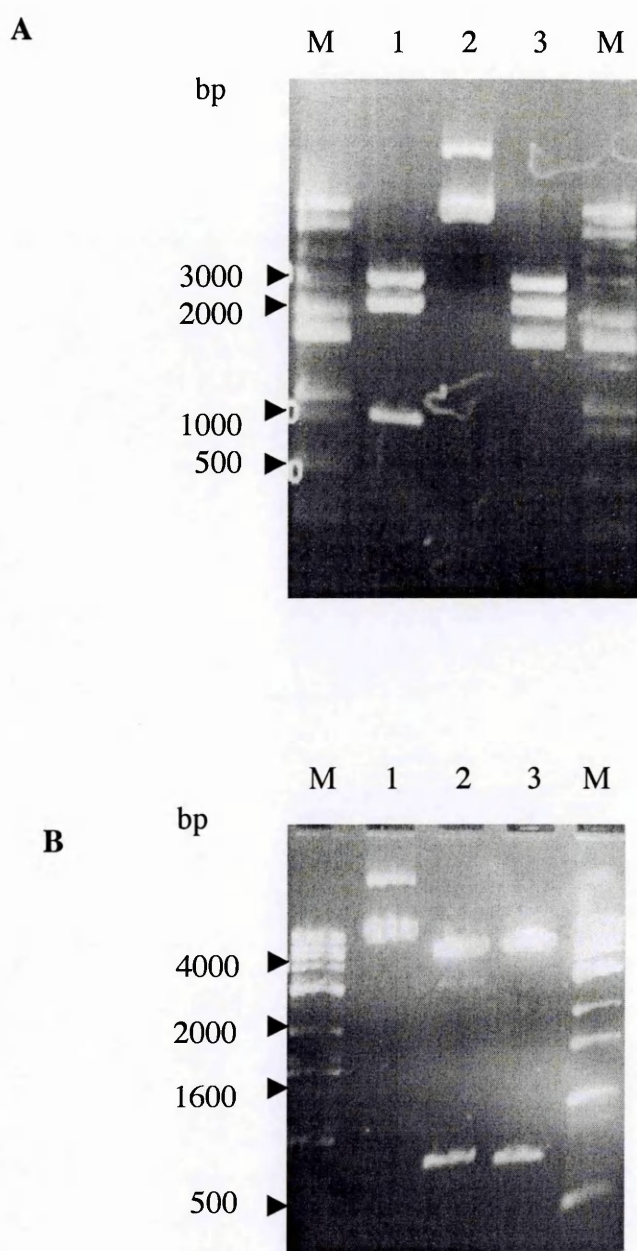


Figure 4.5 Restriction fragment analysis of the final pVAC2-scFv DNA vaccine B05. 1  $\mu$ g of DNA was incubated with 1  $\mu$ l of restriction enzyme and 1  $\mu$ l of reaction buffer at the appropriate temperature for 4 hours. 5  $\mu$ l of each digest was analysed on a 1.5% agarose gel. A) Lane 1 – pVAC2 SalI digest, Lane 2 – pVAC2-scFv uncut, Lane 3 – pVAC2-scFv SalI digest. B) Lane 1 – pVAC2-scFv uncut, Lane 2 – pVAC2-scFv HindIII / NotI digest, Lane 3 – pVAC2-scFv SfiI / NotI digest. M – 1 Kb DNA marker.

| Vaccine Identifier | Expiry Date | DNA conc. (µg/ml) | OD 260 : 280 ratio | Sterility | Endotoxin level EU(IU)/ml |           | Structure/sequence confirmation |          |
|--------------------|-------------|-------------------|--------------------|-----------|---------------------------|-----------|---------------------------------|----------|
|                    |             |                   |                    |           | BPL                       | NIBSC     | digests                         | sequence |
| B02                | 09/2000     | 500               | 2.1                | STERILE   | 1250                      | 600-1200  | ✓                               | ✓        |
| M01 **             | 07/2001     | 495               | 1.9                | STERILE   | <7.8                      | 2.4-4.8   | ✓                               | ✓        |
| M02                | 09/2001     | 500               | 1.9                | STERILE   | <7.8                      | 2.4-4.8   | ✓                               | ✓        |
| S01                | 03/2001     | 500               | 1.9                | STERILE   | 47.9                      | 240-300   | ✓                               | ✓        |
| S02                | 08/2001     | 500               | 1.9                | STERILE   | <7.8                      | 0.6-1.2   | ✓                               | ✓        |
| B01 **             | 11/2001     | 1038              | 1.7                | STERILE   | 10.6                      | 120-240   | ✓                               | ✓        |
| M03                | 11/2001     | 1000              | 2.1                | STERILE   | <7.8                      | 0.3-0.6   | ✓                               | ✓        |
| M04                | 11/2001     | 1021              | 2.0                | STERILE   | <7.8                      | 0.3-0.6   | ✓                               | ✓        |
| S03                | 10/2001     | 1000              | 1.9                | STERILE   | 16.4                      | 38.4-76.8 | ✓                               | ✓        |
| S04                | 11/2001     | 992               | 1.9                | STERILE   | 109                       | nt        | ✓                               | ✓        |
| M05 **             | 01/2002     | 1558              | 1.9                | STERILE   | 52.5                      | 120-240   | ✓                               | ✓        |
| M06                | 09/2002     | 1500              | 1.9                | STERILE   | 775                       | 600-1200  | ✓                               | ✓        |
| S06                | 07/2002     | 1542              | 1.9                | STERILE   | 133.5                     | 240-300   | ✓                               | ✓        |
| S07 **             | 07/2002     | 1489              | 2.0                | STERILE   | <7.8                      | 1.2-2.4   | ✓                               | ✓        |
| S011               | 10/2002     | 1500              | 2.0                | STERILE   | 391.5                     | 300-600   | ✓                               | ✓        |
| B03 **             | 01/2003     | 1980              | 1.9                | STERILE   | 8.4                       | 24-30     | ✓                               | ✓        |
| B04                | 04/2003     | 2080              | 1.8                | STERILE   | 0.106                     | 30-60     | ✓                               | ✓        |
| S09                | 03/2003     | 2080              | 1.8                | STERILE   | 13.8                      | 12-24     | ✓                               | ✓        |
| S012               | 11/2002     | 1963              | 2.0                | STERILE   | <7.8                      | 12-24     | ✓                               | ✓        |
| S013 **            | 04/2003     | 2075              | 1.8                | STERILE   | 8.1                       | 2.4-4.8   | ✓                               | ✓        |
| B05 **             | 08/2003     | 2455              | 1.8                | STERILE   | 14.4                      | 3.0-6.0   | ✓                               | ✓        |
| M08                | 09/2003     | 2480              | 1.7                | STERILE   | 42.2                      | 30-60     | ✓                               | ✓        |
| S05                | 08/2003     | 2500              | 1.9                | STERILE   | 24.8                      | 60-120    | ✓                               | ✓        |
| S017               | 08/2004     | 2455              | 1.8                | STERILE   | 279                       | 240-480   | ✓                               | ✓        |
| S018               | 12/2004     | 2560              | 1.8                | STERILE   | nt                        | 1200-2400 | ✓                               | ✓        |

Table 4.5 Summary of the 25 vaccine preparations. The expiry date, concentration, sterility, endotoxin levels and DNA sequence / structure confirmation of the final products are recorded. \*\* indicates vaccines prepared and checked by N Jennings. nt – not tested.

## Chapter 4B

### Antibody Responses in Rabbits and Follicular Lymphoma Patients receiving pVAC2-scFv DNA vaccines

#### 4.4 Results

##### 4.4.1 Antibody responses in rabbits

Dutch rabbits were immunised intramuscular with 200 µg of pVAC2-scFv at days 0, 14 and 28, bleeds were taken at days 0 and 35. IgG responses against tetanus and idiotype was measured and used to assess transcription and translation of the patient vaccine plasmids *in vivo*. The potential immunogenicity of the vaccine was measured by the ability of the individual rabbits to produce anti-idiotypic responses. However, it is important to note that there were no tests performed for the expression of antigen (patient recombinant scFv). Therefore failure to detect an immune response in the rabbit sera may be due to failure of expression, lack of immunogenicity or inadequate delivery of the scFv.

##### 4.4.1.1 Anti-tetanus

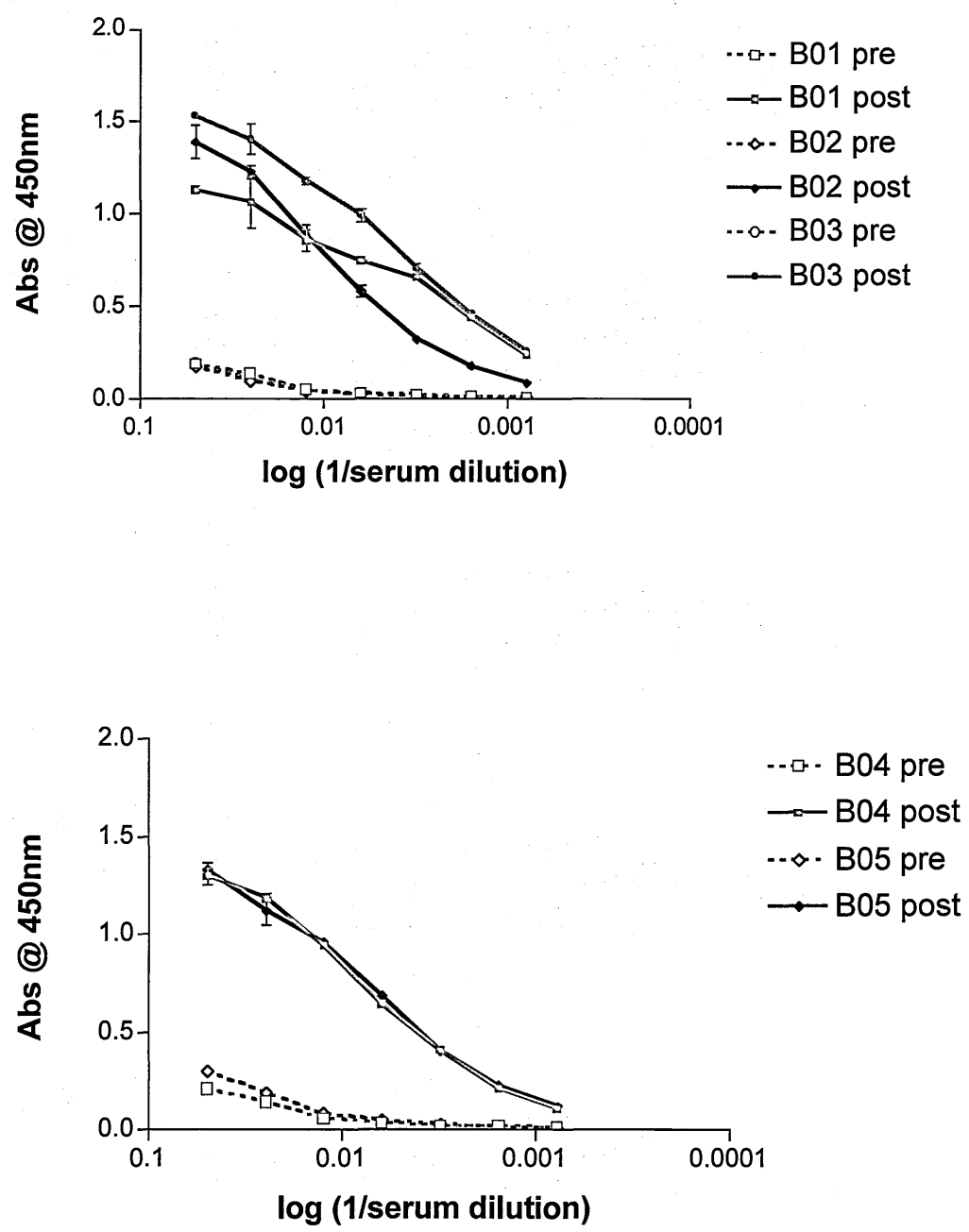
Sera from rabbits immunised with pVAC2-scFv were assayed for the presence of anti-tetanus IgG; pre-immune samples were also assayed as negative controls (Figure 4.6).

ELISA was performed as described in Chpt 2.2.4.1. The anti-tetanus IgG produced varied between vaccines but the majority were able to elicit good to excellent responses after three immunisations (19/23) (Figure 4.6 A-D). The responses were arbitrarily graded based on values for the area under the curves (Table 4.6). The remaining four, M06, M08, S03 and S013 required a further two immunisations in order to produce measurable but low potency anti-tetanus responses (Table 4.6). For 21 of the 23 rabbits tested the IgG levels had titrated out at a dilution of 1/1280. The remaining 2, S09 and S011 required further dilutions (1/20000) to complete the titration curve (Figure 4.6 D).

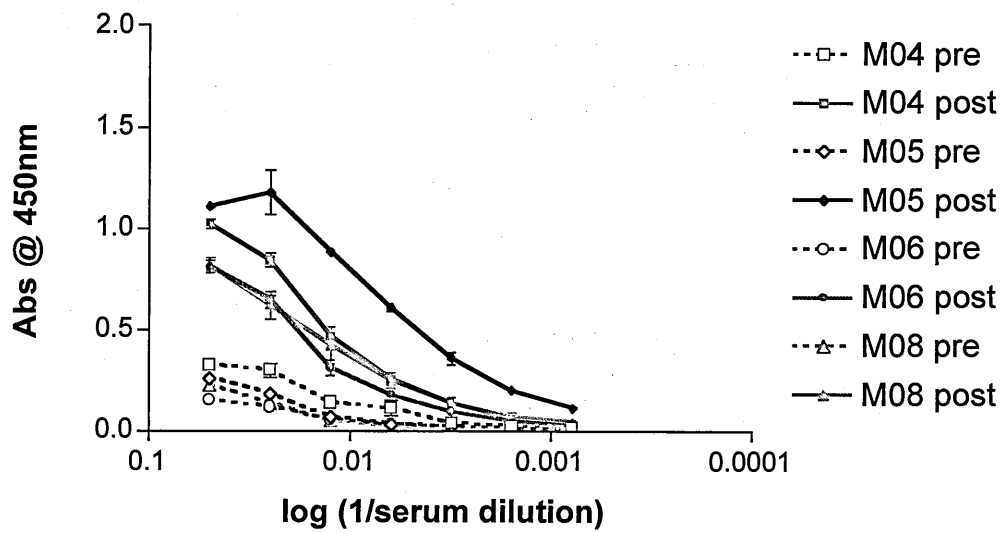
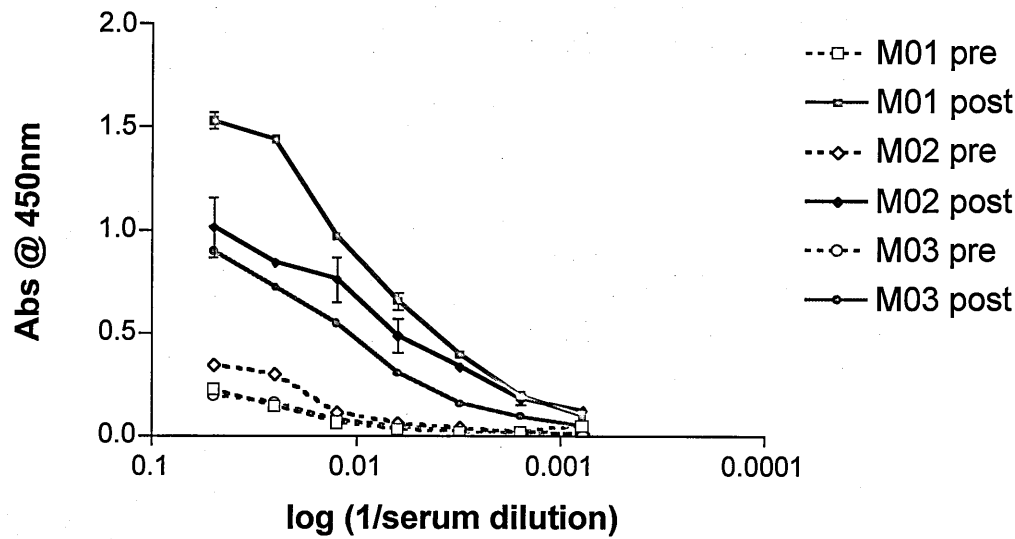
| Vaccine Identifier   | Anti-tetanus response |     | Anti-idiotypic response |     |
|----------------------|-----------------------|-----|-------------------------|-----|
| No. of immunisations | 3                     | 5   | 3                       | 5   |
| B01                  | +++                   |     |                         | +++ |
| B02                  | +++                   |     | +++                     |     |
| B03                  | +++                   |     |                         | +   |
| B04                  | +++                   |     | +++                     |     |
| B05                  | +++                   |     | +++                     |     |
| M01                  | +++                   |     | +++                     |     |
| M02                  | +++                   |     |                         | –   |
| M03                  | ++                    |     | +++                     |     |
| M04                  | +++                   |     | +++                     |     |
| M05                  | +++                   |     | nt                      | nt  |
| M06                  |                       | ++  |                         | +++ |
| M08                  |                       | ++  |                         | +++ |
| S01                  | +++                   |     | +++                     |     |
| S02                  | +++                   |     |                         | +++ |
| S03                  |                       | ++  |                         | –   |
| S04                  | +++                   |     |                         | +++ |
| S05                  | +++                   |     |                         | +++ |
| S06                  | +++                   |     |                         | ++  |
| S07                  | +++                   |     |                         | +   |
| S09                  | +++                   |     | +++                     |     |
| S011                 | +++                   |     | +++                     |     |
| S012                 | +++                   |     |                         | –   |
| S013                 |                       | +++ |                         | ++  |

Table 4.6 Summary of IgG responses in Dutch rabbits receiving pVAC2-scFv. The anti-tetanus and anti-idiotypic was measured after 3 immunisations, a negative response resulted in 2 further immunisations and re-testing. The responses were graded based on values for area under the curve (AUC). Key: AUC > 0.7, ++++ = Excellent; AUC 0.5-0.7, +++ = Good; AUC 0.2-0.5, ++ = Adequate, AUC 0.1-0.2, + = Poor; AUC < 0.1, no response.

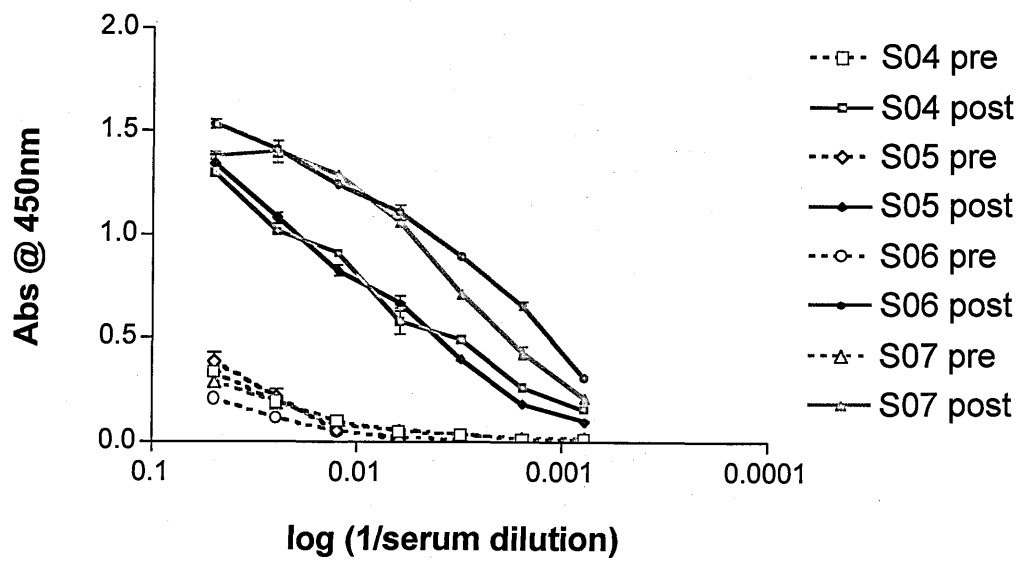
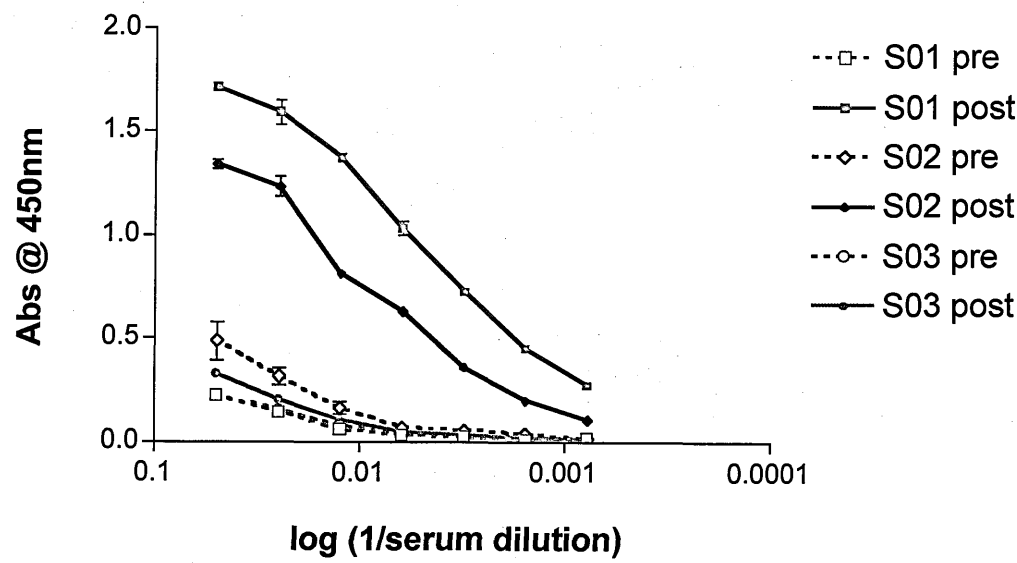
Figure 4.6 A) Anti-tetanus responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



B) Anti-tetanus responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



C) Anti-tetanus responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.





D) Anti-tetanus responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.

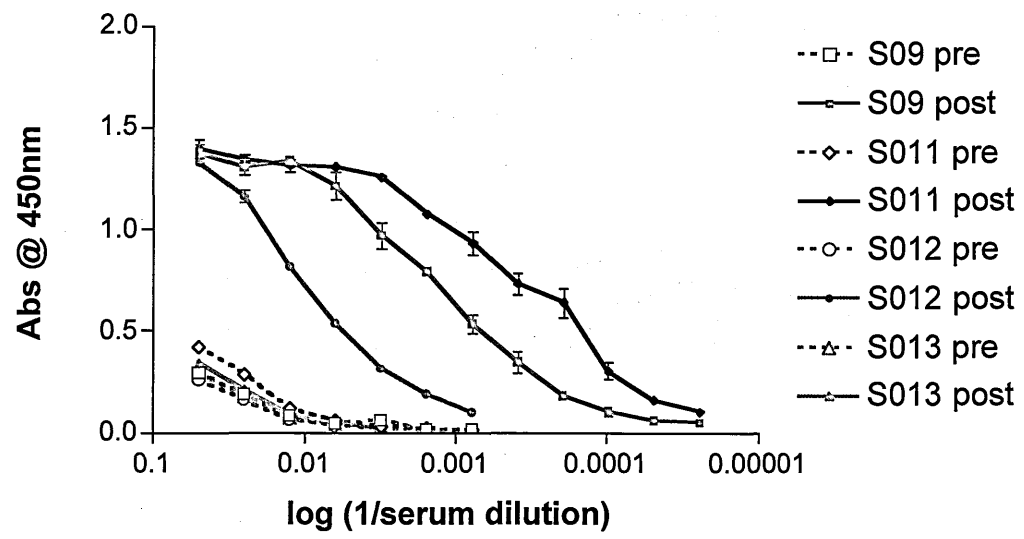
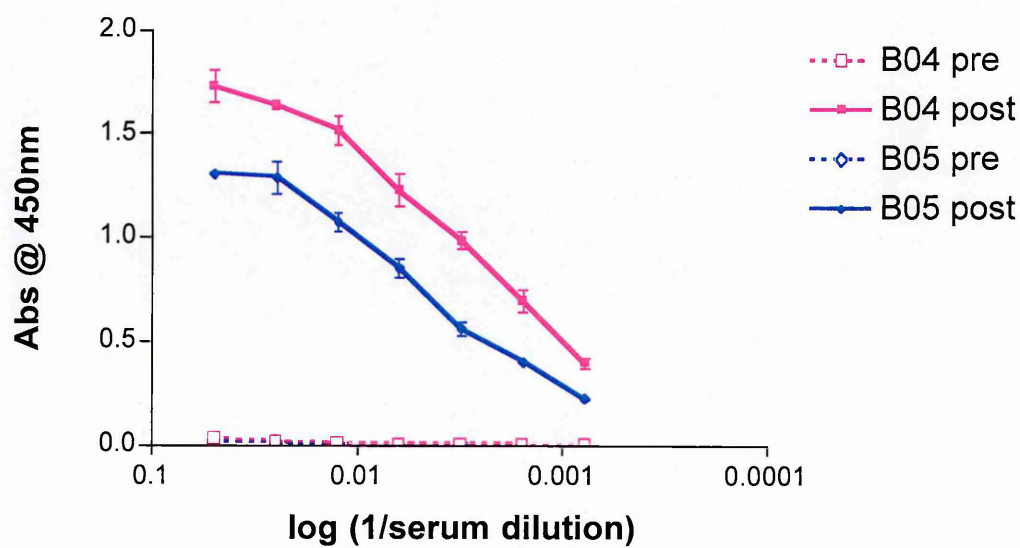
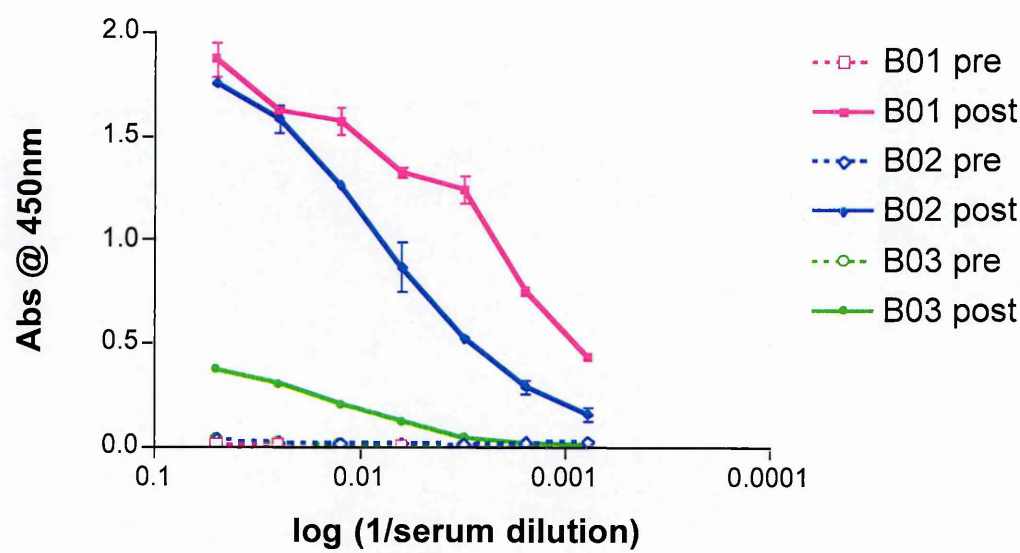


Figure 4.6 A-D Anti-tetanus responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines. Each line represents a titration of pre-immune (pre, broken line) or immune (post, solid line) serum from a single rabbit. Animals were immunised i.m. into both quadriceps, with two doses of 100  $\mu$ g of patient DNA vaccine on days 0, 14 and 28. An ear vein bleed was taken on days 0 and 35 and tested in ELISA. The responses are shown as specific IgG present in immune compared to pre-immune serum samples.

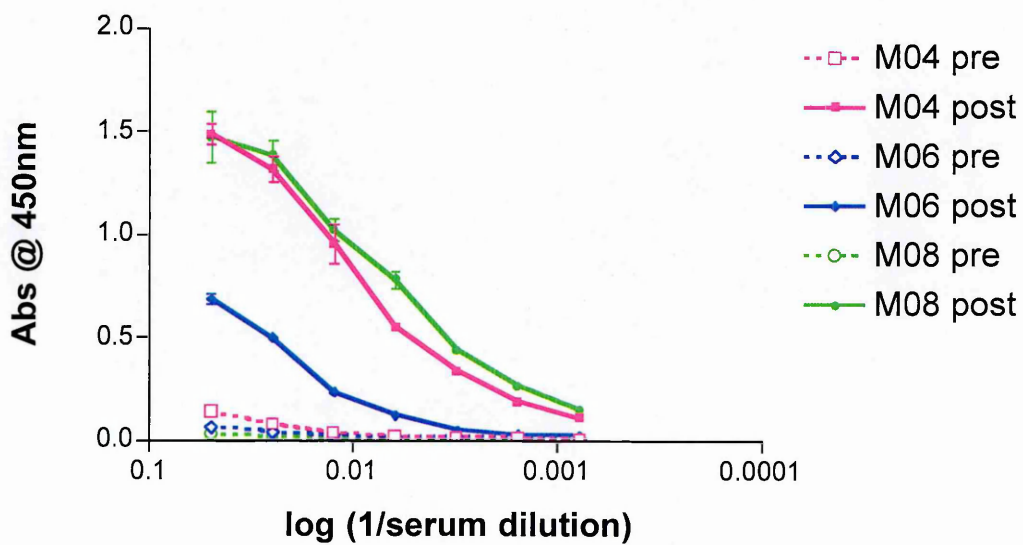
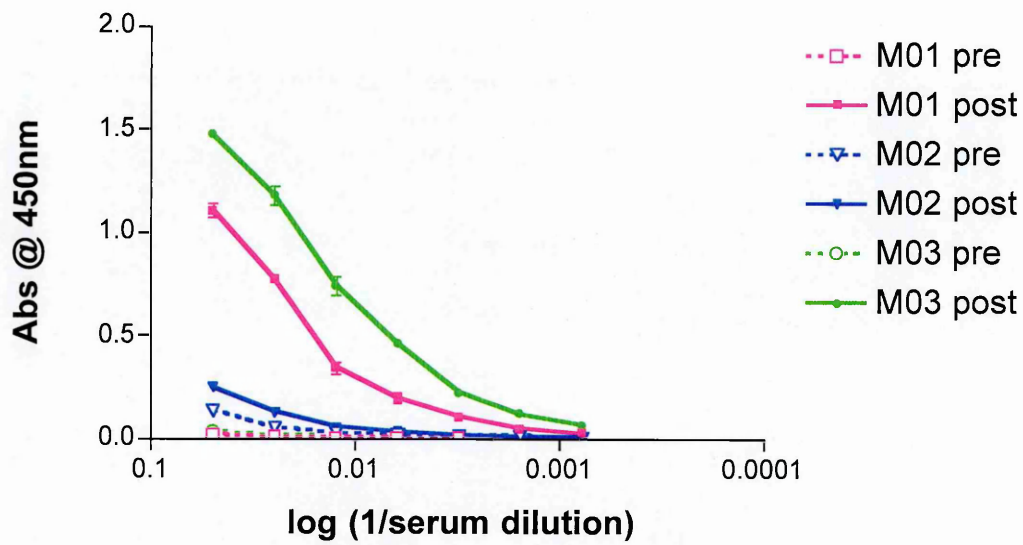
#### 4.4.1.2 Anti-idiotypic

ELISA was performed as described in Chpt 2.2.4.2. The responses of anti-id between the rabbits were varied. Nine vaccines elicited good to excellent responses after three immunisations. The remaining thirteen received a further two immunisations; resulting in a good to excellent response in a further six rabbits and four achieved poor to adequate responses (Table 4.6). Of the remaining five vaccines, B03, S07 and S012 produced a poor response and M02 and S03 still failed to elicit an anti-id response (Figure 4.7 B, C and D). Interestingly the poor response to vaccination for three vaccines (M02, S03 and S012) was related with poor expression of the respective scFvs in *Drosophila* S2 cells (Table 3.1). Anti-idiotypic responses to vaccine M05 could not be measured as no recombinant scFv was produced but a rabbit anti-Tet response was observed with this vaccine (Figure 4.6 B). The responses were graded as before based on values for area under the curve. As with the anti-tetanus responses 21 of the 22 sera tested titrated out beyond the dilution of 1/1280. Due to the high titres of anti-tetanus for S09 and S011 these samples were titrated further than the other samples. The anti-idiotypic titration for S09 was similar to that of the other anti-idiotypes titrating out at 1/2560. S011 however titrated out at 1/20480.

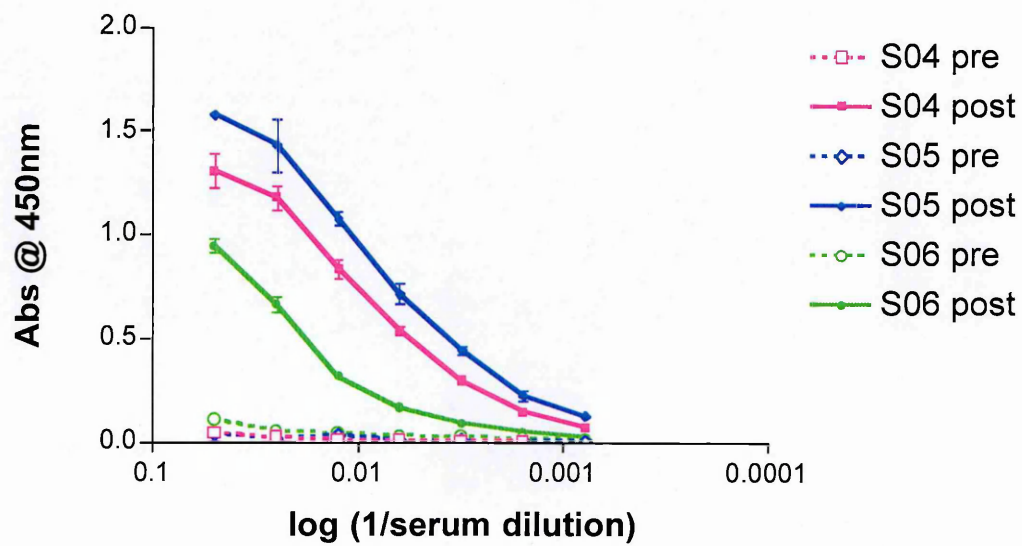
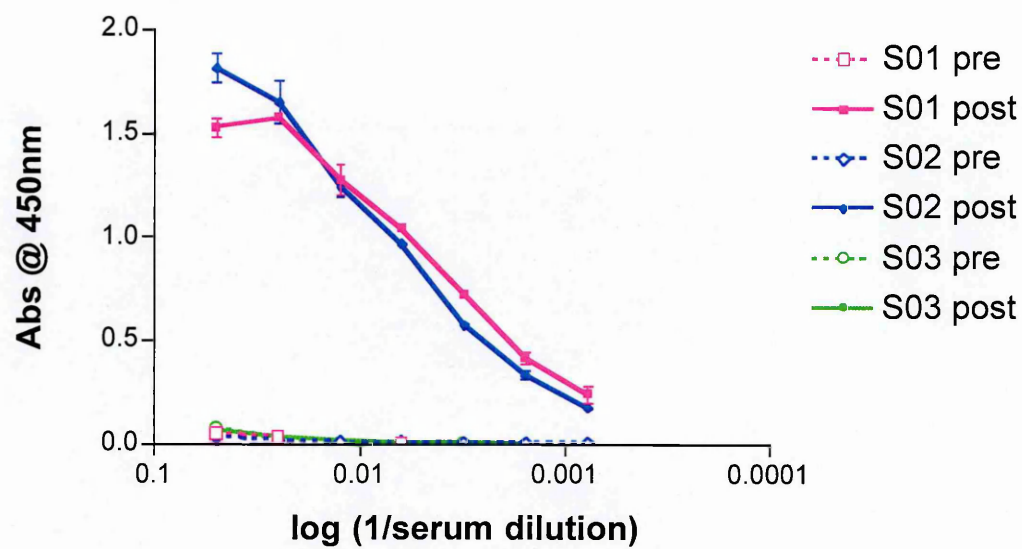
Figure 4.7 A) Anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



B) Anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



C) Anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



D) Anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.

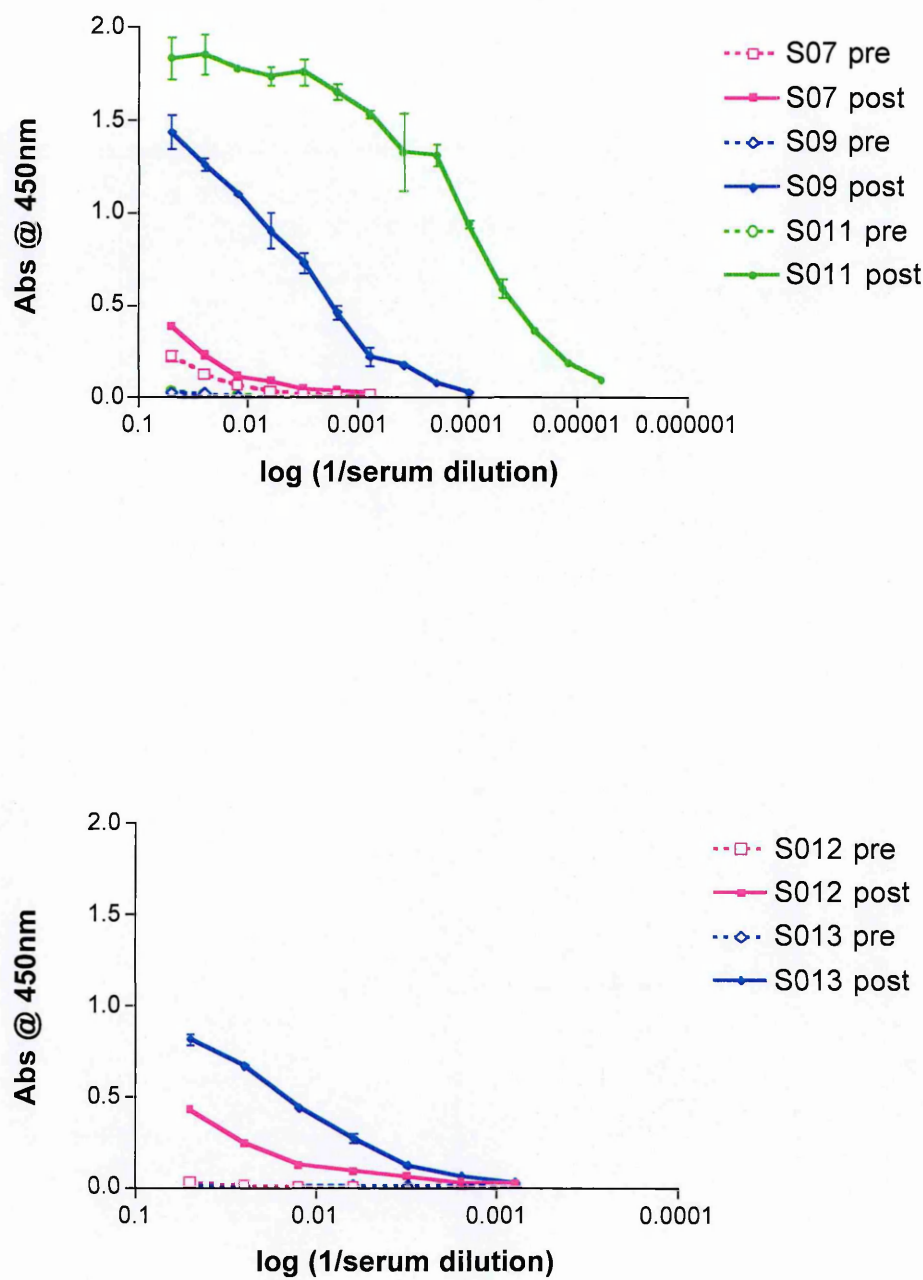







Figure 4.7 A-D Anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines. Each line represents a titration of pre-immune (pre, broken line) or immune (post, solid line) serum from a single rabbit. Animals were immunised i.m. into both quadriceps, with two doses of 100 µg of patient DNA vaccine on days 0, 14 and 28. An ear vein bleed was taken on days 0 and 35 and tested by the CaM-scFv capture ELISA. The responses are shown as specific IgG present in immune compared to pre-immune serum samples.

#### 4.4.2 Cross reactivity of anti-idiotypes in rabbit serum

Polyclonal anti-idiotypic responses are prone to cross-reactivity (reviewed in (Davie *et al.*, 1986)) possibly as the direct result of the sharing of V<sub>H</sub> or V<sub>L</sub> domains between cognate and non-cognate idiotypes. In this chapter the level of cross-reactivity has been investigated in some detail using the unique panel of serum samples from 23 Dutch rabbits immunised with FrC enhanced DNA vaccines harboring unique V<sub>H</sub>/V<sub>L</sub> gene cassettes. The anti-idiotypic activity against the cognate idiotypic was determined in all samples (Table 4.7) and the 17 rabbit sera with good to excellent activity against the cognate idiotypic were entered in further studies to investigate cross-reactivity. Excluded from the analysis were the rabbit serum samples that failed to give a good (minimum ++) response to cognate scFv (Table 4.6) and the four for which recombinant expression of scFv proved awkward. The experiment for cross-reactivity will also establish whether any of the rabbit sera has activity against the common amino-terminal Arg-Ser-Met-Ala and the carboxy-terminal Ala triplet motifs introduced into the recombinant protein by the NcoI and NotI restriction sites respectively or the CaM tag. The experiment was therefore limited to 17 recombinant FL patient derived scFv-CaM and the prototype scFv-B2 and its corresponding rabbit anti-Id serum, which was used to produce a titration curve for each ELISA plate. In the first instance cross-reactivity was performed using each of the 18 rabbit serum samples in turn against the panel of 18 recombinant CaM tagged scFvs. Five of the serum samples tested were cross-reactive; a summary of these findings is presented in Table 4.7.

| scFv                                 | B01            | B02    | B04    | B05  | M01    | M03    | M04   | M06    | M08        | S01    | S02   | S04   | S05   | S06   | S09   | S011   | S013   | B2     |
|--------------------------------------|----------------|--------|--------|------|--------|--------|-------|--------|------------|--------|-------|-------|-------|-------|-------|--------|--------|--------|
| V <sub>H</sub> gene family           | 4              | 3      | 3      | 3    | 4      | 3      | 1     | 3      | 3          | 4      | 3     | 3     | 3     | 3     | 3     | 4      | 3      | 1      |
| Closest germline V <sub>H</sub> gene | 4-59           | P1     | 3-48   | 3-23 | 4-39   | 3-23   | 1-02  | 3-23   | 3-15       | 4-34   | 3-21  | 3-21  | 3-48  | 3-30  | 3-48  | 4-34   | 3-48   | 1-02   |
| V <sub>L</sub> gene family           | λ7             | κ4     | κ3     | κ1   | κ4     | λ2     | κ1    | κ4     | λ2         | κ3     | λ1    | κ3    | κ1    | κ1    | κ1    | κ4     | κ3     | λ2     |
| Closest germline V <sub>L</sub> gene | 14-7           | DPK 24 | DPK 22 | A30  | DPK 24 | DPL 11 | DPK 1 | DPK 24 | 2c. 118 D9 | DPK 22 | DPL 2 | Vg 38 | DPK 9 | DPK 9 | DPK 9 | DPK 24 | DPK 22 | DPL 11 |
| Serum                                | Germline       |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |
| B01                                  | 4-59<br>λ 14-7 |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |
| M04                                  | 1-02<br>DPK 1  |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |
| S05                                  | 3-48<br>DPK 9  |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |
| S09                                  | 3-48<br>DPK 9  |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |
| S011                                 | 4-34<br>DPK 24 |        |        |      |        |        |       |        |            |        |       |       |       |       |       |        |        |        |

Table 4.7 Summary of the ELISA experiments to test for cross-reactivity. The VH and VL gene families of each clone are presented in rows 2 and 4. The VH and VL gene sequences were aligned against the V gene germline sequences at the nucleotide level and the closest homologue is presented in rows 3 and 5. The five cross-reactive rabbit sera are shown. The responses were calculated as a percentage of the cognate. Key: > 75%  ; > 50% < 75%  ; > 25% < 50%  ; < 25%  ; no reactivity .



13 of the 18 serum samples do not cross react, they are specific for the cognate idiotype that was used for immunisation. Five of the serum samples (B01, M04, S05, S09 and S011) did cross-react with 6, 3, 9, 3 and 7 scFvs respectively. For all these the extent of cross-reactivity was further quantitated by testing the reaction with the non-cognate scFvs in titration (Figures 4.8 A-D). The cross-reacting serum was titrated to establish the strength of the cross reactivity (Chpt 2.2.4.2). Based on this additional analysis the antisera can be divided in two types of narrow and broad cross-reactivity. M04 and S09 are examples of the former and the remaining three of the latter (Table 4.7).

M04 only reacts with three of the 17 scFvs and two of these (S05 & S09) have a high degree of homology to the same germline  $V_H$  gene V3-48 and the same germline  $V_L$  gene DPK9 ( $V_{\kappa 1}$ ). The antiserum also reacts with B05, a scFv with a similar  $V_H$  domain encoded by the  $V_{H3}$  family gene 3-23 and a  $V_{\kappa 1}$  family gene A30. As expected the antiserum obtained by immunisation with vaccine S05 does react with scFv M04 but the one obtained by S09 immunisation does not. The latter is another example of an antiserum with narrow cross-reactivity and anti-S09 sees only three additional clones (S04, S05 and S013). Two of the three (S05 and S013) carry  $V_H$  genes with homology to V3-48 paired with the  $V_{\kappa 1}$  and  $V_{\kappa 3}$  domains homologous to DPK9 and DPK22 respectively. The  $V_H$  domain shows 85% homology to the one used for immunisation. The third reactive scFv is S04 (encoded by germline genes 3-21/ $V_{g38}$ ) with a  $V_H$  and  $V_{\kappa}$  domain belonging to the same gene family groups as the other two reactive idiotypes.

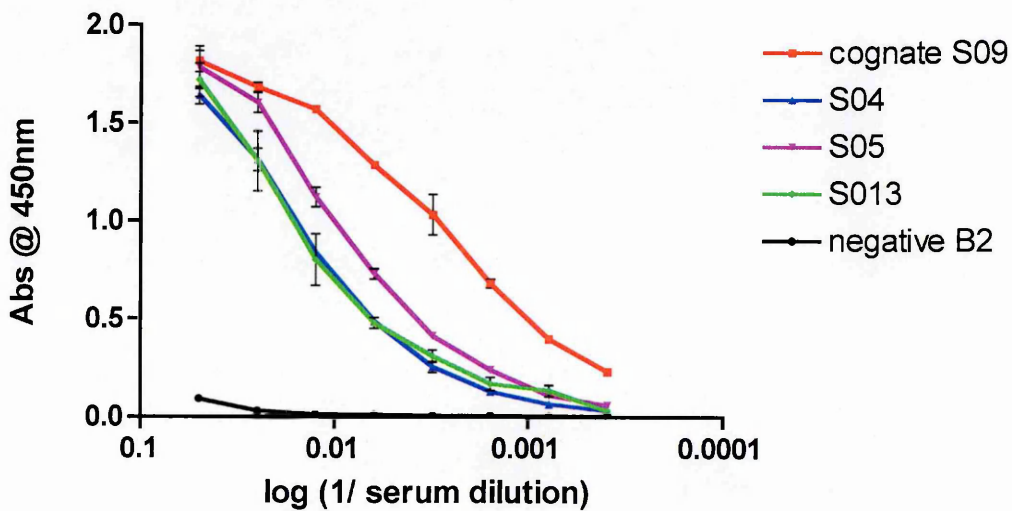
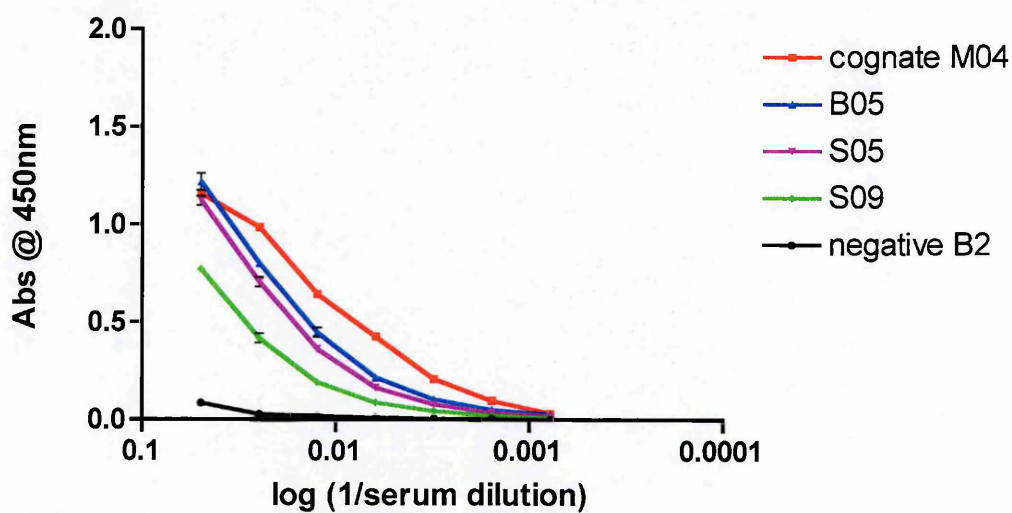
The antisera against B01, S05 and S011 have rather broad cross reactivity. On initial observation the pattern of cross-reactivity is poorly explained but a more careful inspection shows some striking similarities between several of the cross-reactivities. For anti-B01 the most reactive scFvs, M01, S01 and S011 all have a V<sub>H</sub> domain encoded by a V<sub>H</sub>4 family gene either paired with DPK22 (S01) or DPK24 (M01, S011). The three other reactive scFvs M08, S02 and B02 are of structurally different categories but all three have a V<sub>λ</sub> domain, either from the V<sub>λ</sub>2 or V<sub>λ</sub>3 gene family. For one of the six reactive scFvs (S011) the corresponding S011 antiserum was also shown to be strongly cross-reactive with scFv B01 confirming the presence of a shared idiotype between the 4-59 and 4-34 germline V<sub>H</sub> domains.

The anti-S05 serum cross-reacts with 9 of the 17 scFvs, three of which B04, S09 and S013 are all encoded by the V3-48 V<sub>H</sub> germline homologous to the V<sub>H</sub> domain of the immunogen. As expected the anti-S09 reacts strongly with scFv S05. The other anti-S05 reactivities are more complex. Phylogenetic analysis of the 27 V<sub>H</sub>3 genes reveals three groups of 10, 14 and 3 genes respectively. Interestingly the 3-15, 3-21 and 3-23 (germlines of M08, S02 and S04 and B05 respectively) are clustered in the second group of 14. Anti-S05 binds the 3-23 strongly when paired with the A30 V<sub>κ</sub>1 domain (B05), but not at all when paired with the V<sub>λ</sub>2 domain DPL11 (M03) or the V<sub>κ</sub>4 domain DPK24 (M06).

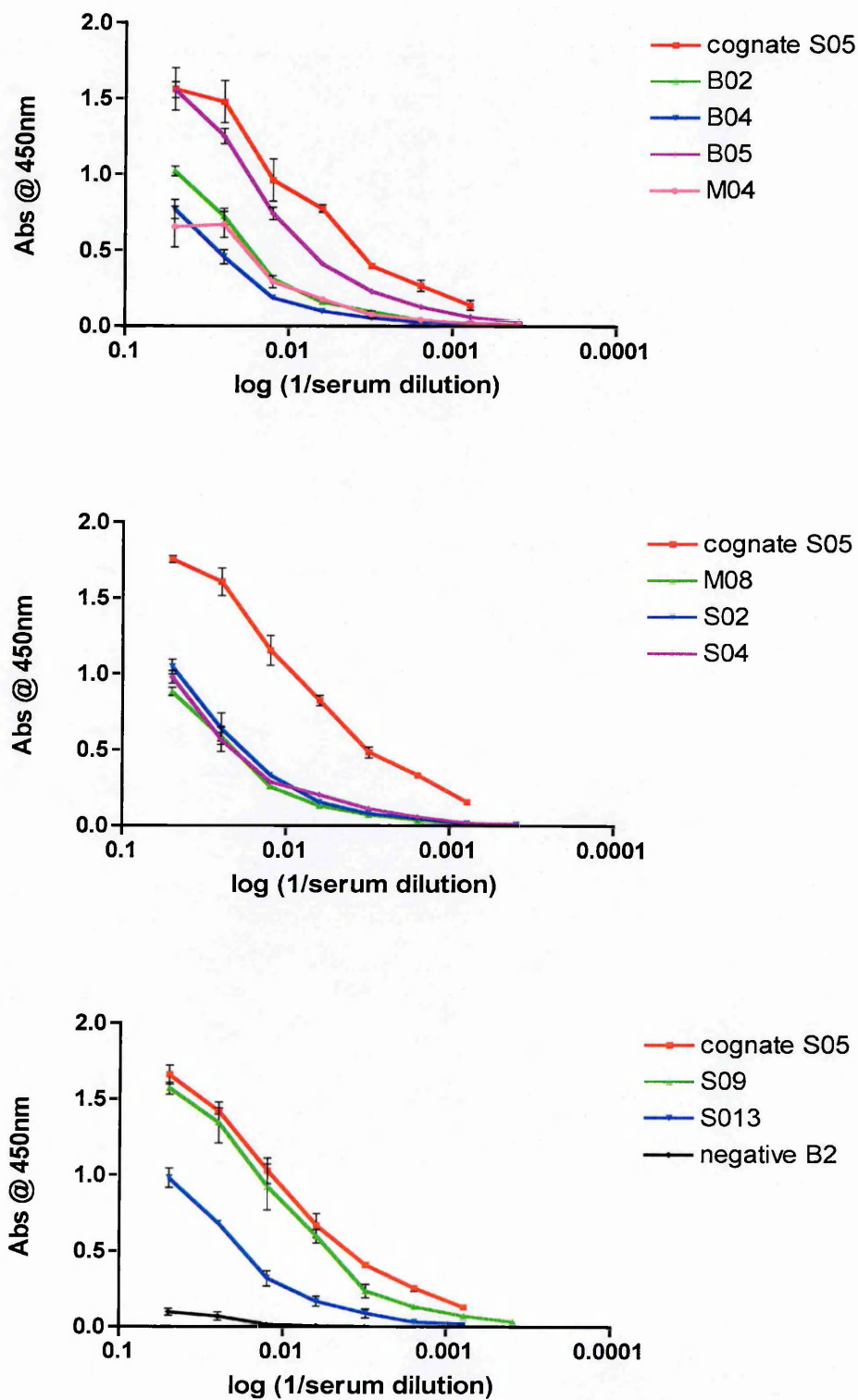
In total four (B04, S05, S09 and S013) antibodies have a 3-48 V<sub>H</sub> domain and all are paired with a V<sub>κ</sub> domain. Interestingly all three 'S' ones share idiotypes defined by anti-S05 and -S09 and two of them (S05 and S013) by anti-S011, but the same three sera are inert (S09 and S011) or weak (S05) against scFv B04. Amino acid sequences

of the  $V_H$  and  $V_k$  domains of S05, S09 and B04 antibodies are shown aligned to their closest germline genes in Figures 4.9 and 4.10. As expected there is a high degree of homology between the  $V_H$  domains (Figure 4.9). The most notable difference is the length of the CDR3 of S09, 15 amino acids compared with the 10 amino acids of S05 and B04. The  $V_L$  genes of S05 and S09 also align to the same  $V\kappa 1$  germline gene, DPK9 whereas the  $V_L$  gene of B04 aligns with a  $V\kappa 3$  germline gene DPK22 (Figure 4.10).

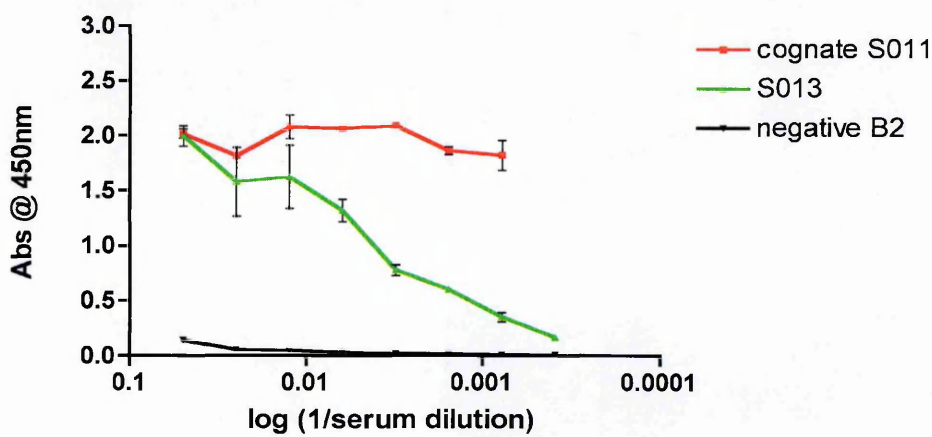
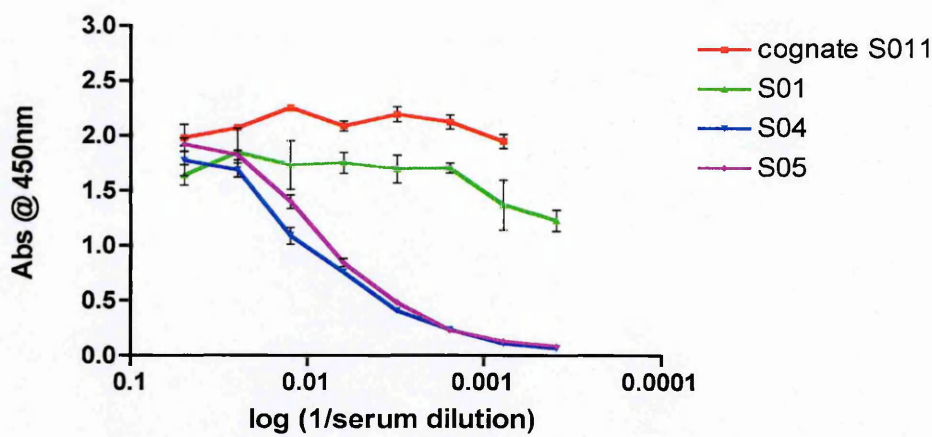
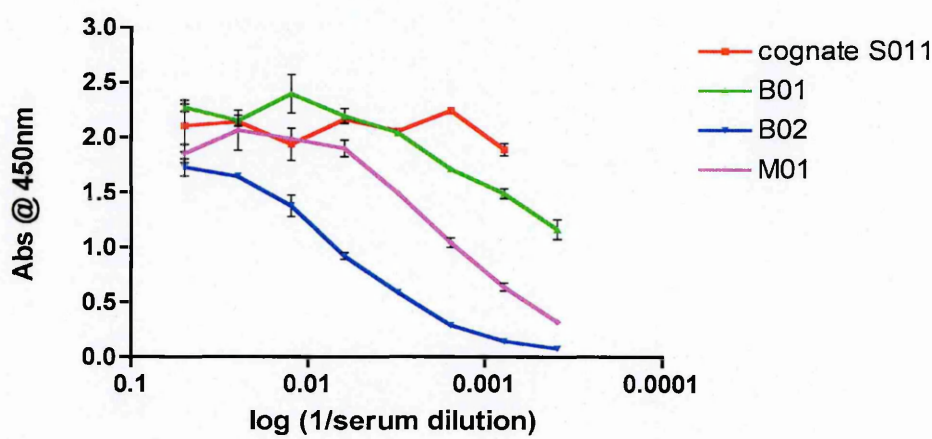
Figure 4.8 A) Cross-reactive anti-idiotypic responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



B) Cross-reactive anti-idiotypic responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



C) Cross-reactive anti-idiotypic responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.



D) Cross-reactive anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines.

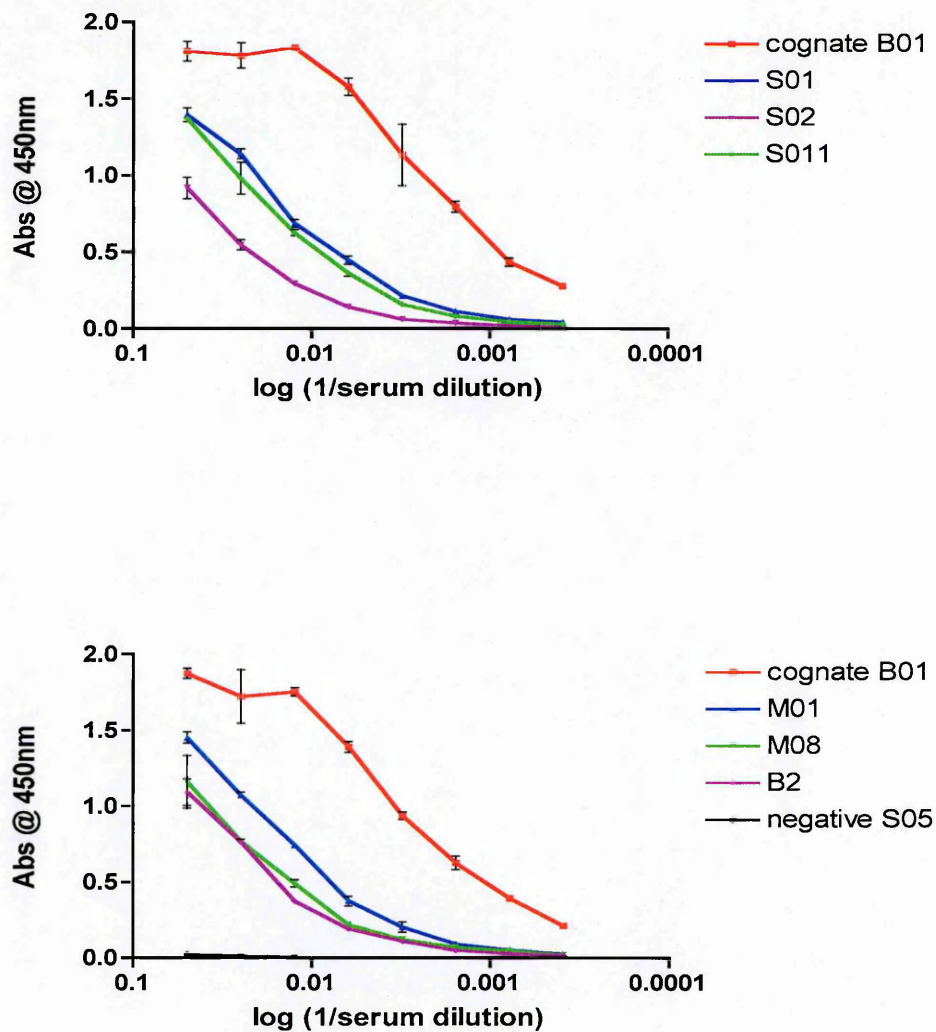


Figure 4.8 A-D Cross-reactive anti-idiotype responses in Dutch rabbits immunised with pVAC2-scFv patient DNA vaccines. Each line represents a titration of post immune serum from a single rabbit. The cognate is shown in red and the negative control in black. Animals were immunised i.m. into both quadriceps, with two doses of 100  $\mu$ g of patient DNA vaccine on days 0, 14 and 28. An ear vein bleed was taken on days 0 and 35 and tested by the CaM-scFv capture ELISA. The responses are shown as specific IgG present in immune serum samples.

|       | FR1   | CDR1           | FR2                                | CDR2                                  |
|-------|---|----------------|------------------------------------|---------------------------------------|
| V3-48 | 1.....10.....20.....30<br> <br>EVQLVESGGGLVQPGGSLRLSCAASGFTFS | .....<br>SYSMN | ....40.....<br> <br>WVRQAPGKGLEWVS | 50.....60....<br> <br>YISSSSTIYYADSVK |
| S05   | -----<br>-L-----  | -----T         | -----                              | N-----N-AF-----                       |
| S09   | -----V-----N-T  | N-N-S          | -----I-                            | ---NT-TS-----                         |
| B04   | ---V---RW-----V-----S-N                                       | A-T-N          | -----R---I-                        | N-T---GF-KD-----                      |

|       | FR3  | CDR3                            | FR4                     |
|-------|--|---------------------------------|-------------------------|
| V3-48 | ....70.....80.....90....<br> <br>GRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR | ....100.....<br> <br>DRNGYNFFDY | 103<br> <br>WGQGTLLTVSS |
| S05   | -----V-----H---D-----F---  | RNVTTVNLGLDVFDI                 | WGQGTMTTVSS             |
| S09   | ---I-----E-----S---D-----  | QNFGGYVFDF                      | WGQGTMTTVSS             |
| B04   | ---V-----G-KN-----L---V-   |                                 |                         |

Figure 4.9 Amino acid sequences of the VH genes encoding S05, S09 and B04 are shown aligned to the closest germ-line gene V3-48. Identical amino acids are illustrated by dashes. Replacement mutations are indicated in uppercase. Numbering according to Kabat.



|       |                                |                |                  |             |
|-------|--------------------------------|----------------|------------------|-------------|
|       | FR1                            | CDR1           | FR2              | CDR2        |
|       | 1.....10.....20...<br>         | .....30...<br> | .....40.....<br> | 50.....<br> |
| DPK9  | DIQMTQSPSSLASVGDRVITC          | RASQSISSYLN    | WYQQKPGKAPKLLIY  | AASSLQS     |
| S05   | --E-----I--L-----              | ---A-RESVQ     | -----MF          | ST-I-E-     |
| S09   | -----V--                       | ---N-N---      | --L---V-----F    | -----       |
|       | FR3                            | FR4            |                  |             |
|       | ...60.....70.....80.....<br>   | .....102<br>   |                  |             |
| DPK9  | GVPSRFGSGGTDFTLTISLQPEDFATYYC  | QQSYSTP        | FGPGTKVEMR       |             |
| S05   | -----F-H-----S-                | --N--G-FT      | FGQGTNVEIN       |             |
| S09   | -----Q-A-----NN-----S---       | -----S-SWT     |                  |             |
|       | FR1                            | CDR1           | FR2              | CDR2        |
|       | 1.....10.....20...<br>         | .....30...<br> | .....40.....<br> | 50.....<br> |
| DPK22 | EIVLTQSPGTLSLSPGERATLSC        | RASQSVSSSYLA   | WYQQKPGQAPRLLIY  | GASSRAT     |
| B04   | -----P-----TNN---              | -----          | ---I-----S       | -----       |
|       | FR3                            | FR4            |                  |             |
|       | ...60.....70.....80.....<br>   | .....102<br>   |                  |             |
| DPK22 | GIPDRFGSGGTDFTLTISRLEPEDFAVYYC | QQYGSSP        | FGQGTNVEIK       |             |
| B04   | -----A-----                    | ---Y-RT        |                  |             |

Figure 4.10 Amino acid sequences of the VL genes encoding S05, S09 and B04 are shown aligned to the closest germ-line genes DPK 9 and 22 respectively. Identical amino acids are illustrated by dashes. Replacement mutations are indicated in uppercase. Numbering according to Kabat

#### **4.4.3 Anti-tetanus responses in normal human serum**

Serum from a random sample of ten donors from the National Blood Service Cambridge donor clinic was assayed for the presence of anti-tetanus IgG (Figure 4.11) (Chpt 2.2.4.1). The samples tested show varied IgG responses against the TEFT coated onto the ELISA plate (Figure 4.11).

#### **4.4.4 Anti-idiotypic responses in normal human serum**

The same panel of 11 donor serum samples was investigated for the presence of anti-idiotypic (Chpt 2.2.4.2). The ELISA data shows that anti-idiotypes are present in random serum samples, with some having broad reactivity and others being more specific, for example samples 4 and 6 (Table 4.8, Figure 4.12). Of the eleven scFvs only M01 was not recognized by any of the donor samples, conversely M03, B04 and B2 were recognized to some degree by all the samples. The remaining seven scFvs were selectively recognized by a small number of donor samples (Table 4.8).

There is no evidence for the presence of antibodies against common epitopes introduced during recombinant scFv expression. However, as discussed in Chapter 3, several of the recombinant scFvs are glycosylated (Table 3.1). B04, M02, M04, M06 and S011 were discovered to bind concanavalin A, which indicates the presence of N-linked glycosylation. Human serum has naturally occurring antibodies against non-self carbohydrate moieties and can therefore explain the wide reactivity observed with some of the scFvs, for example B04. Site-directed mutagenesis has been used on B04 and B05 to remove the N-linked glycosylation sites (Chpt 4.2.3).

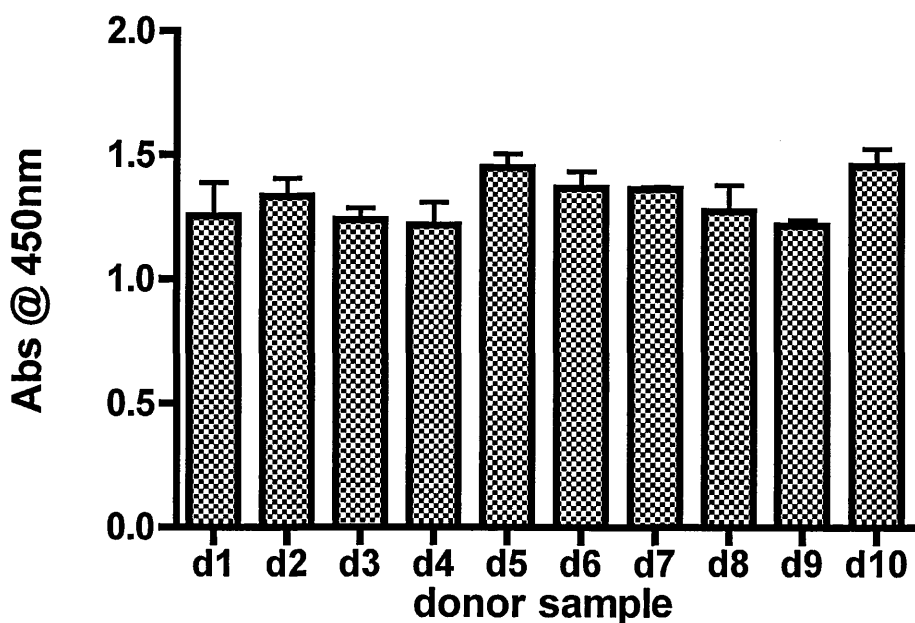


Figure 4.11 Anti-tetanus in normal human serum. Each bar represents a 1:20 serum dilution from an individual donor. Serum was taken with the donors consent from a routine visit to the donor clinic at the National Blood Service Cambridge. Samples were tested for the presence of anti-tetanus by ELISA. The results are shown as bound IgG present in each sample.

| Donor | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|-------|---|---|---|---|---|---|---|---|---|----|----|
| scFv  |   |   |   |   |   |   |   |   |   |    |    |
| B03   |   |   |   |   |   |   |   |   |   |    |    |
| B04   |   |   |   |   |   |   |   |   |   |    |    |
| B05   |   |   |   |   |   |   |   |   |   |    |    |
| M01   |   |   |   |   |   |   |   |   |   |    |    |
| M02   |   |   |   |   |   |   |   |   |   |    |    |
| M03   |   |   |   |   |   |   |   |   |   |    |    |
| M04   |   |   |   |   |   |   |   |   |   |    |    |
| M06   |   |   |   |   |   |   |   |   |   |    |    |
| M08   |   |   |   |   |   |   |   |   |   |    |    |
| S011  |   |   |   |   |   |   |   |   |   |    |    |
| B2    |   |   |   |   |   |   |   |   |   |    |    |






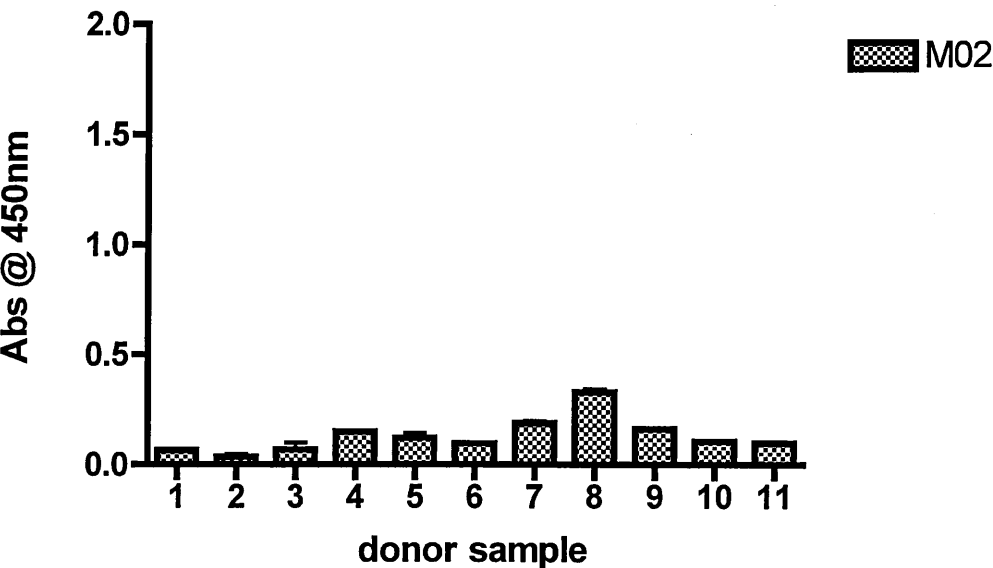
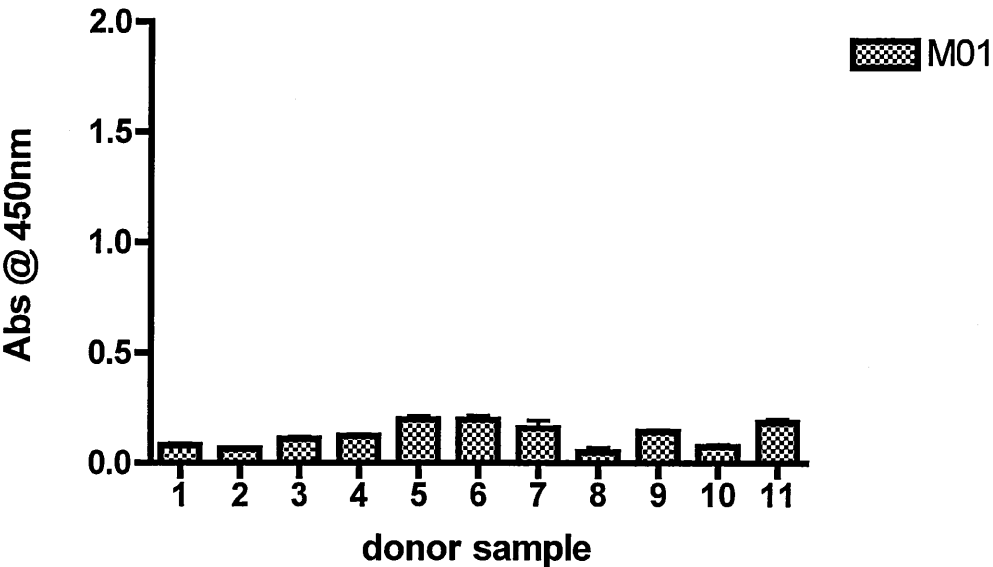
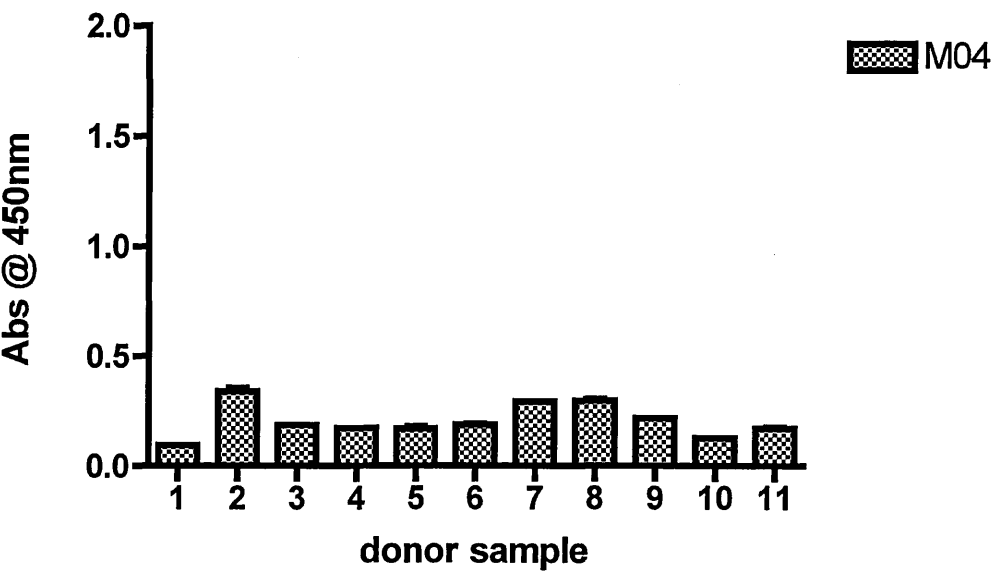
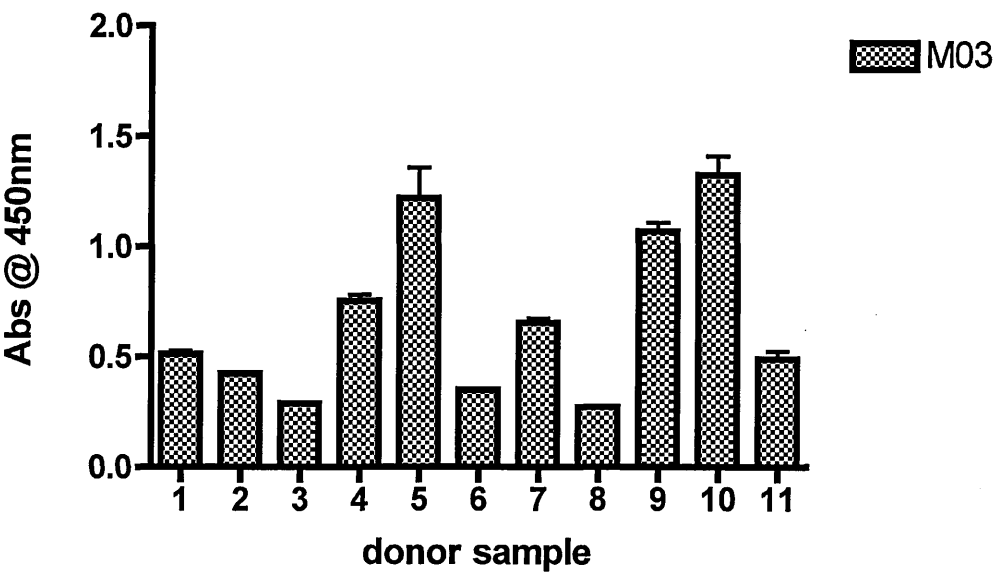
Table 4.8 Summary of the ELISA data assessing the anti-idiotypic binding in control human serum samples. The OD value @ 450nm was taken and the responses quantified as follows; OD > 1.5 , OD > 1.0 < 1.5 , OD > 0.5 < 1.0 , OD > 0.25 < 0.5 , OD < 0.25 .

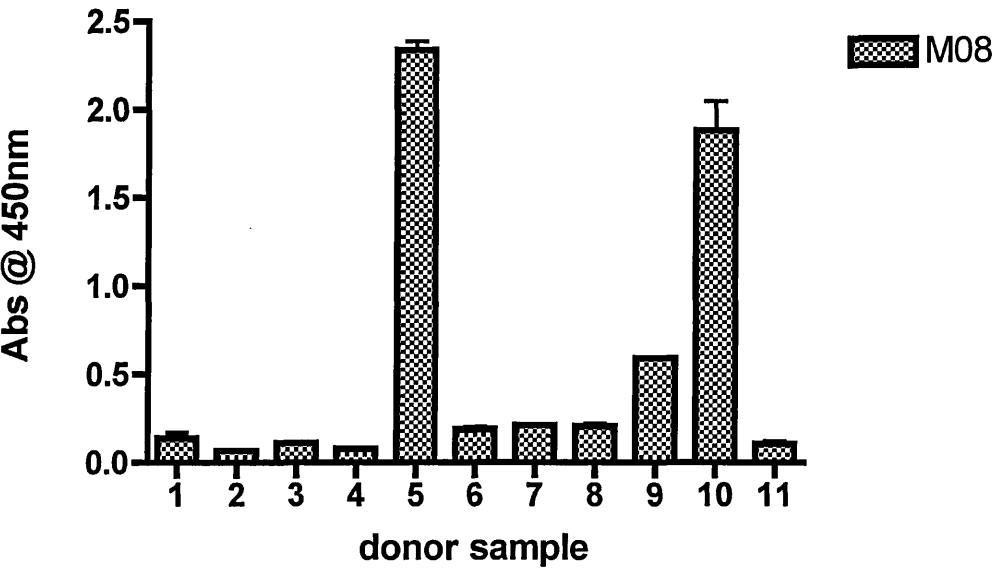
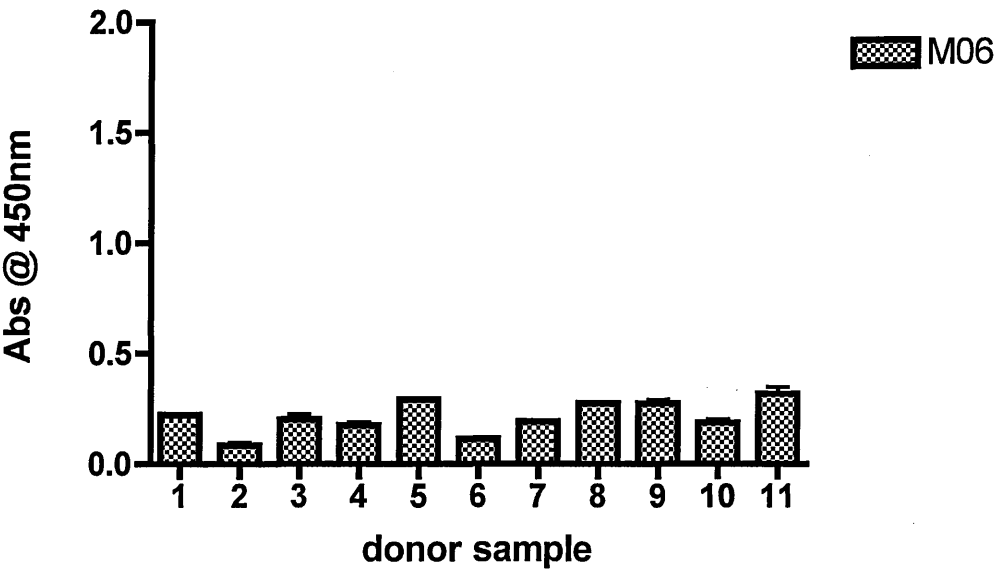
Figure 4.12 A) Anti-idiotypic activity in control human serum samples.



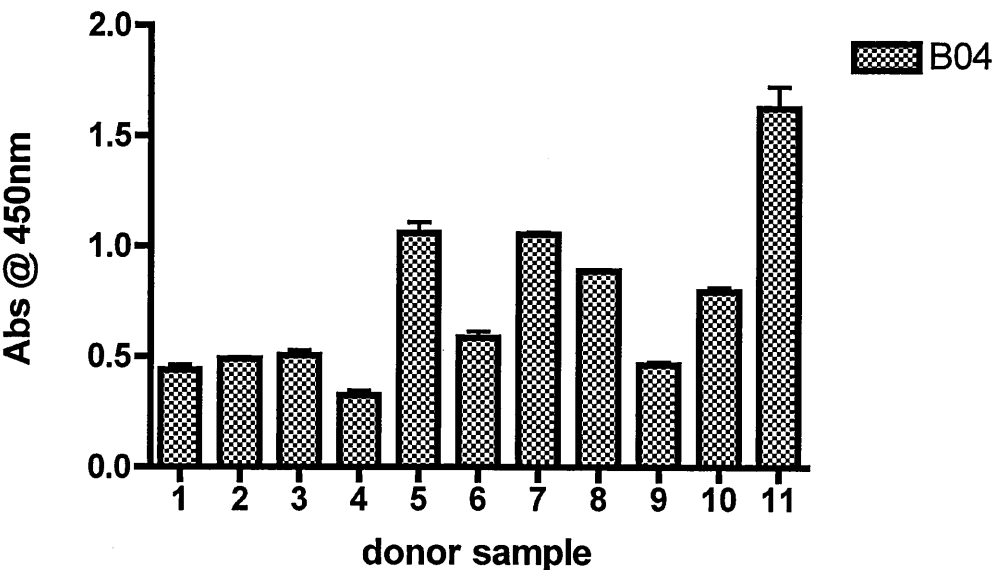
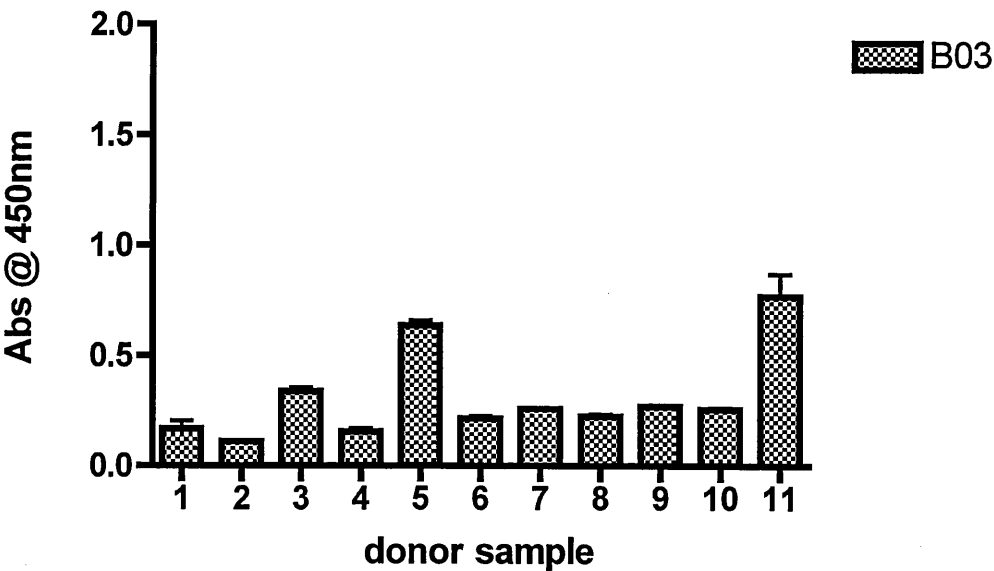
B) Anti-idiotype activity in control human serum samples.



C) Anti-idiotype activity in control human serum samples.

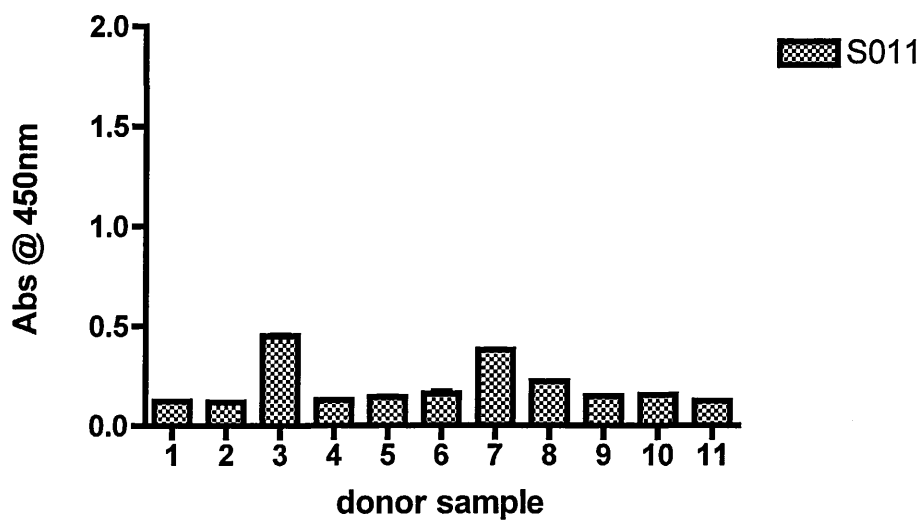
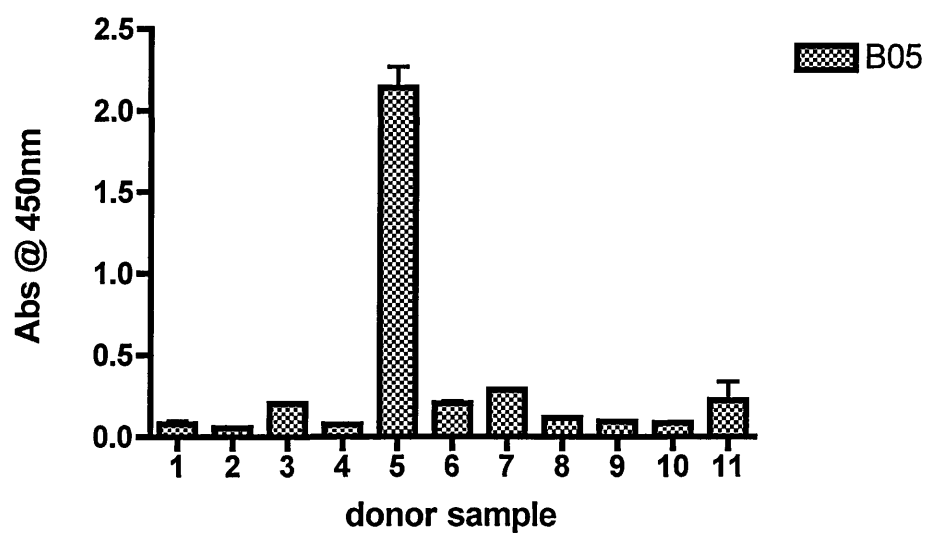


D) Anti-idiotype activity in control human serum samples.





E) Anti-idiotypic activity in control human serum samples.



F) Anti-idiotype activity in control human serum samples.

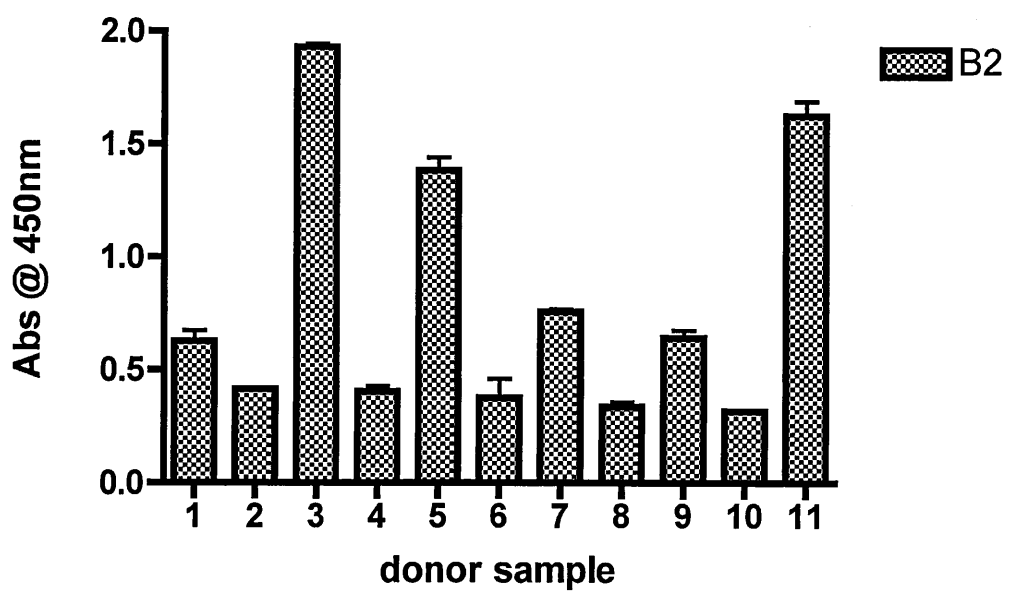


Figure. 4.12 A-F Anti-idiotype activity in normal human serum. Each bar represents a 1:20 serum dilution from an individual donor. Serum was taken with the donors consent at a routine visit to the donor clinic at NBS Cambridge. Samples were tested for the presence of anti-idiotype by the CaM-scFv capture ELISA. The responses are shown as specific bound IgG.

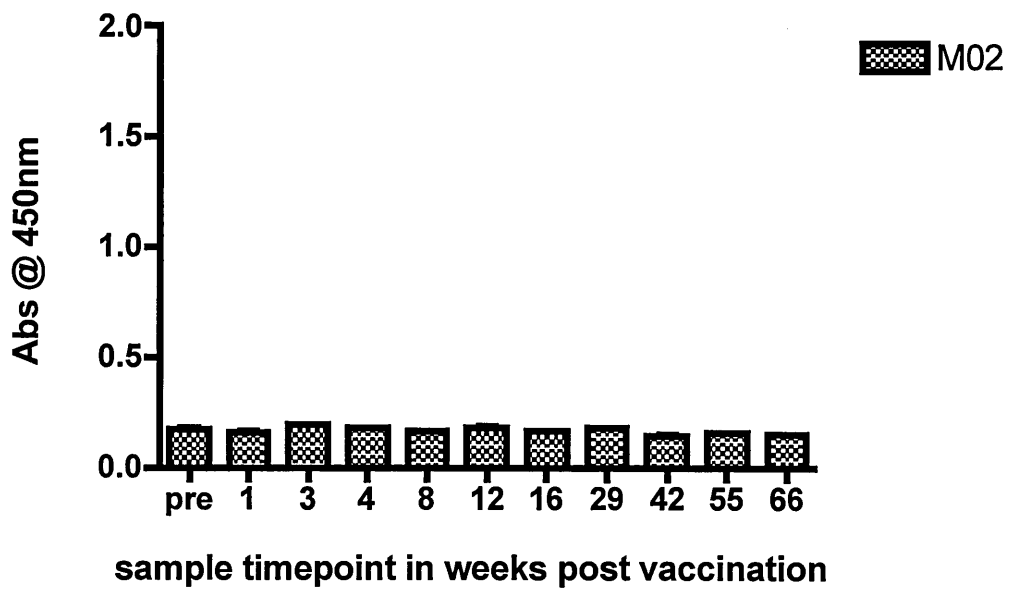
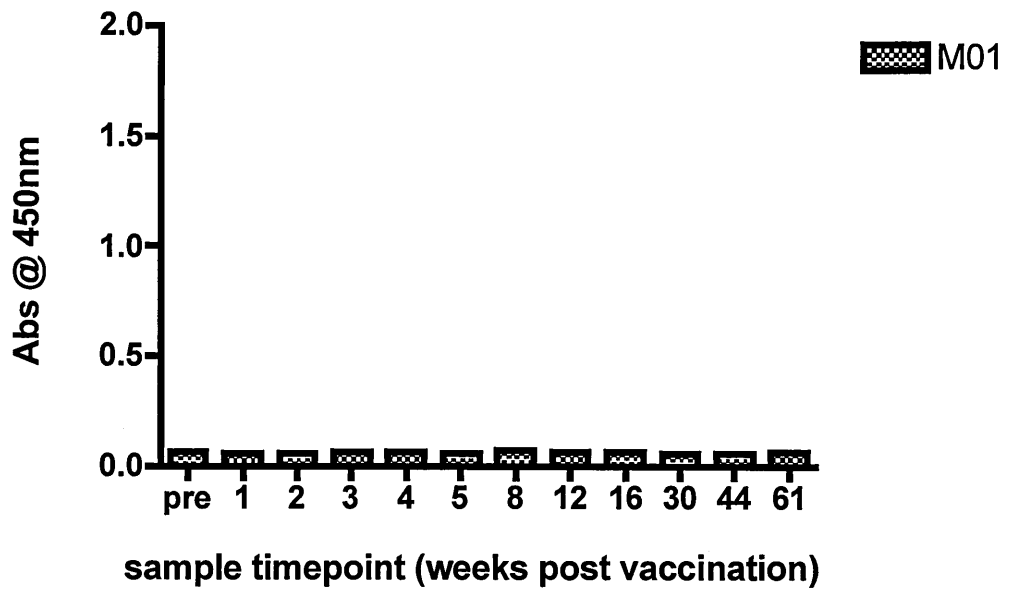
#### **4.4.5 Anti-idiotypic responses in patients' pre and post vaccination with pVAC2-scFv**

The serum samples from selected patients were tested for the presence of IgG against their cognate scFvs by ELISA (Figure 4.13). Each patient sample was used at a dilution of 1:20, and results with B03, M01, M02, M04, M06 and M08 are shown in Figure 4.13 A, B and C. From this we conclude that there is no measurable IgG response against cognate scFv either pre- or post-immunisation. Patients B04, B05, M03 and S011 (Figure 4.13 D, E) have measurable IgG responses against cognate scFv, however there is no increase in this response post-immunisation. The pre- and final post-immunisation samples from each patient were tested against the extracellular domains of GPVI (D1D2-CaM) as a negative control. The OD at 450 nm for each sample was less than 0.2 (data not shown).

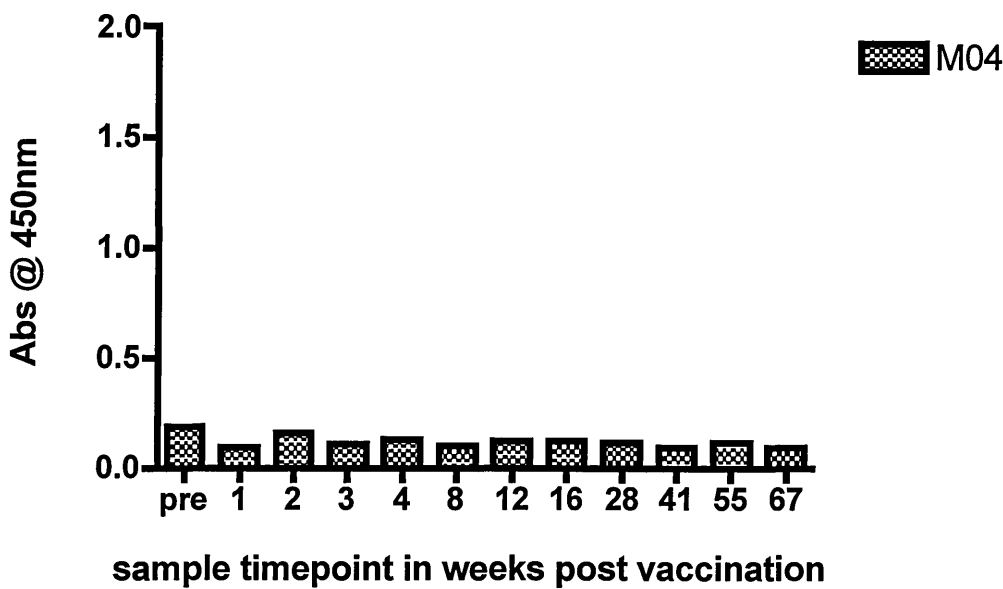
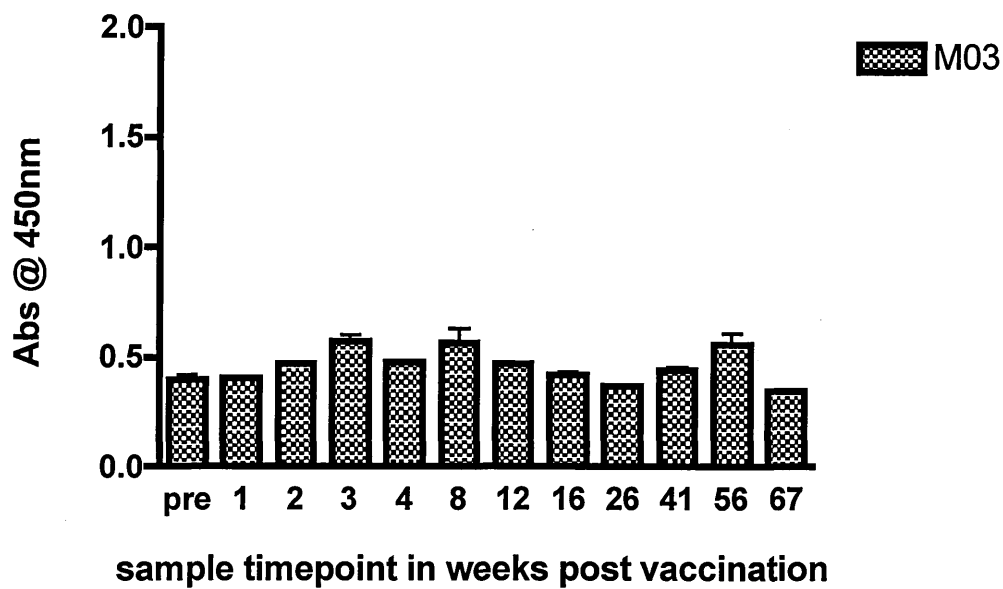
#### **4.4.6 Anti-tetanus responses in patients pre and post vaccination with pVAC2-scFv**

The patient serum samples that were positive for anti-idiotypic were tested for the presence of anti-tetanus (Figure 4.14). There is no significant difference in the IgG responses against TEFT in the pre- and post-immune samples. Also there is no significant difference to the anti-tetanus in these patients compared with the donor control panel (Figure 4.11).

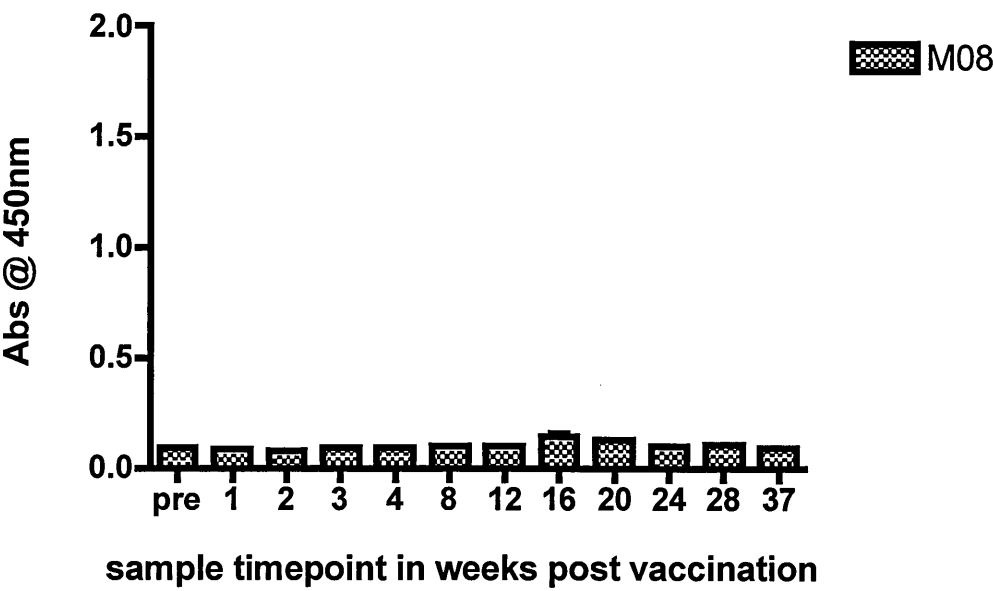
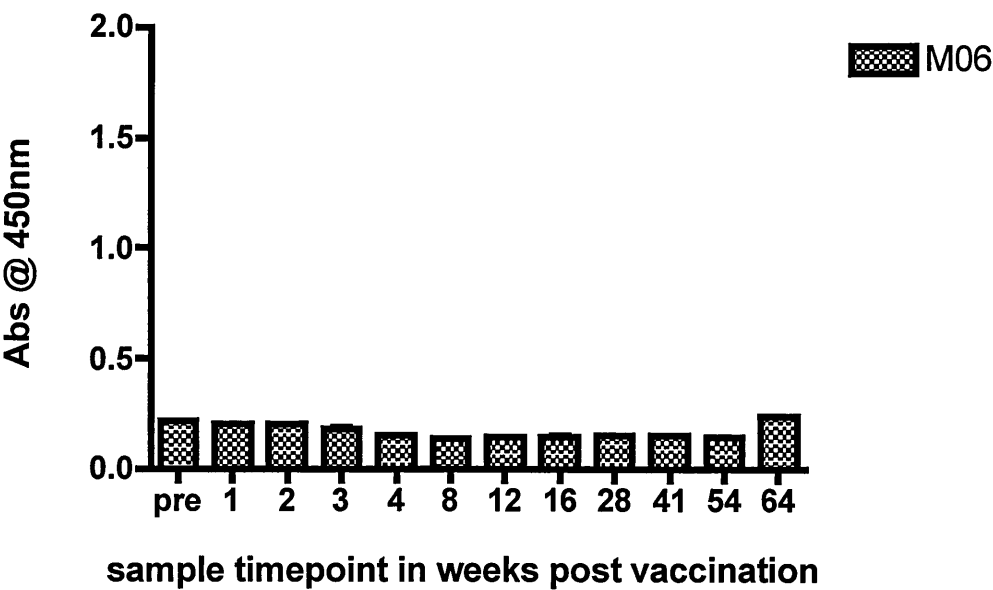
Figure 4.13 A) Anti-idiotypic activity against cognate scFv in sequential patient samples.



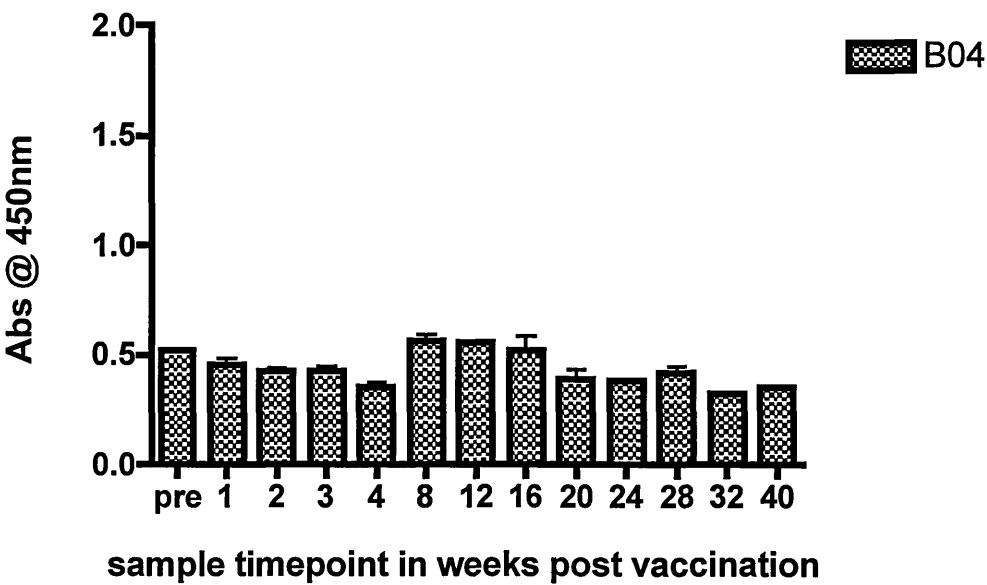
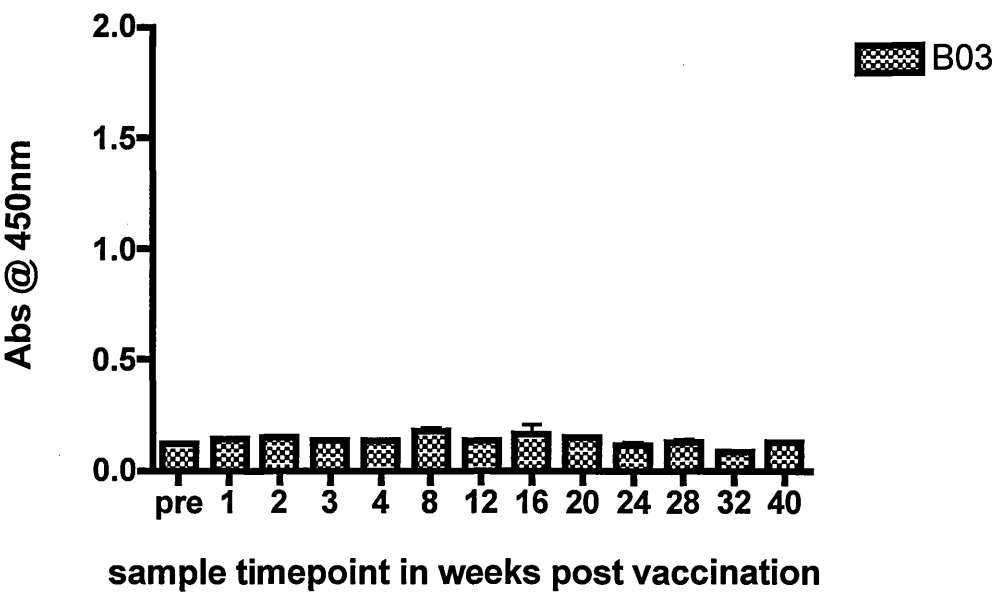
B) Anti-idiotype activity against cognate scFv in sequential patient samples.



C) Anti-idiotypic activity against cognate scFv in sequential patient samples.



D) Anti-idiotypic activity against cognate scFv in sequential patient samples.



E) Anti-idiotype activity against cognate scFv in sequential patient samples.

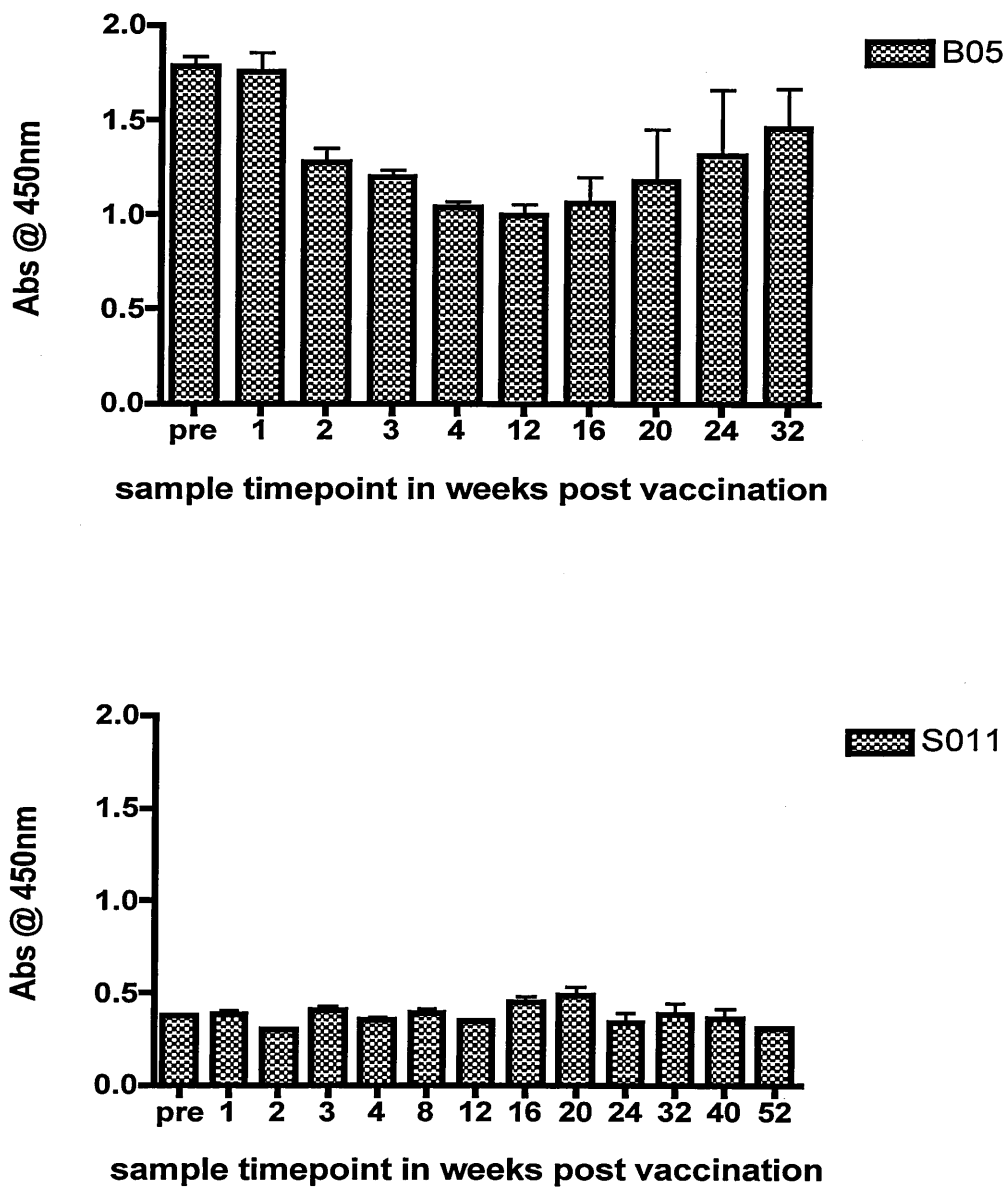
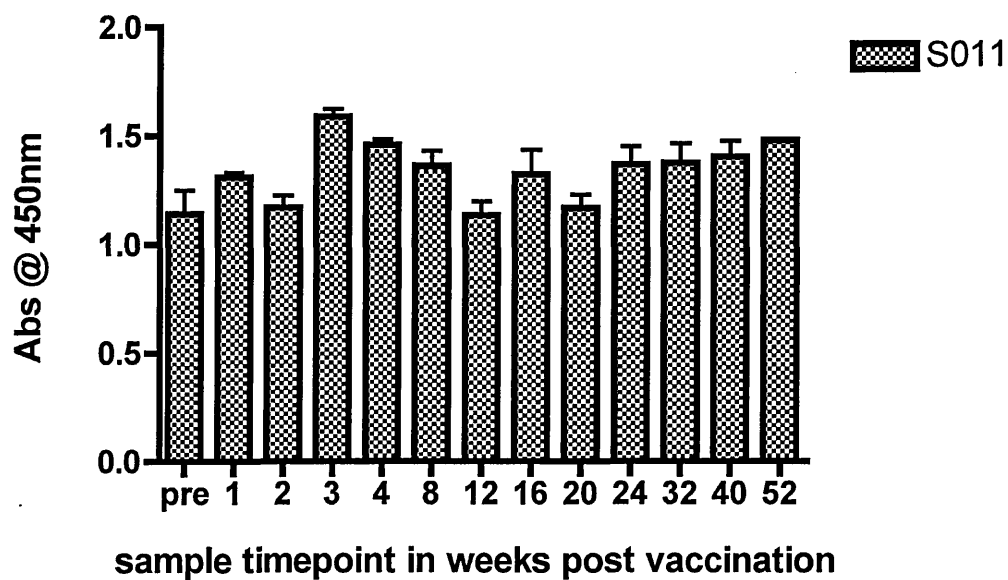
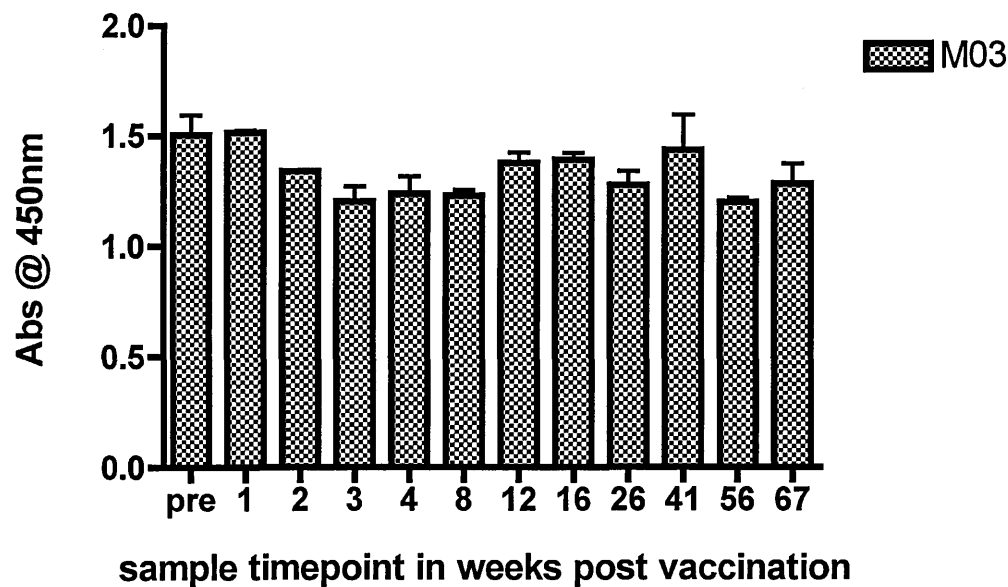


Figure 4.13 A-E Anti-idiotype activity against cognate scFv in sequential patient samples. Several serum samples from selected patients were tested for the presence of specific IgG against cognate idiotyp by the CaM-scFv capture ELISA. The responses are shown as specific bound IgG. Each bar represents a 1:20 serum dilution from each individual, the timepoint of sampling in relation to the first vaccination is presented.



Figure 4.14 A) Anti-tetanus activity in sequential patient samples.



B) Anti-tetanus activity in sequential patient samples.

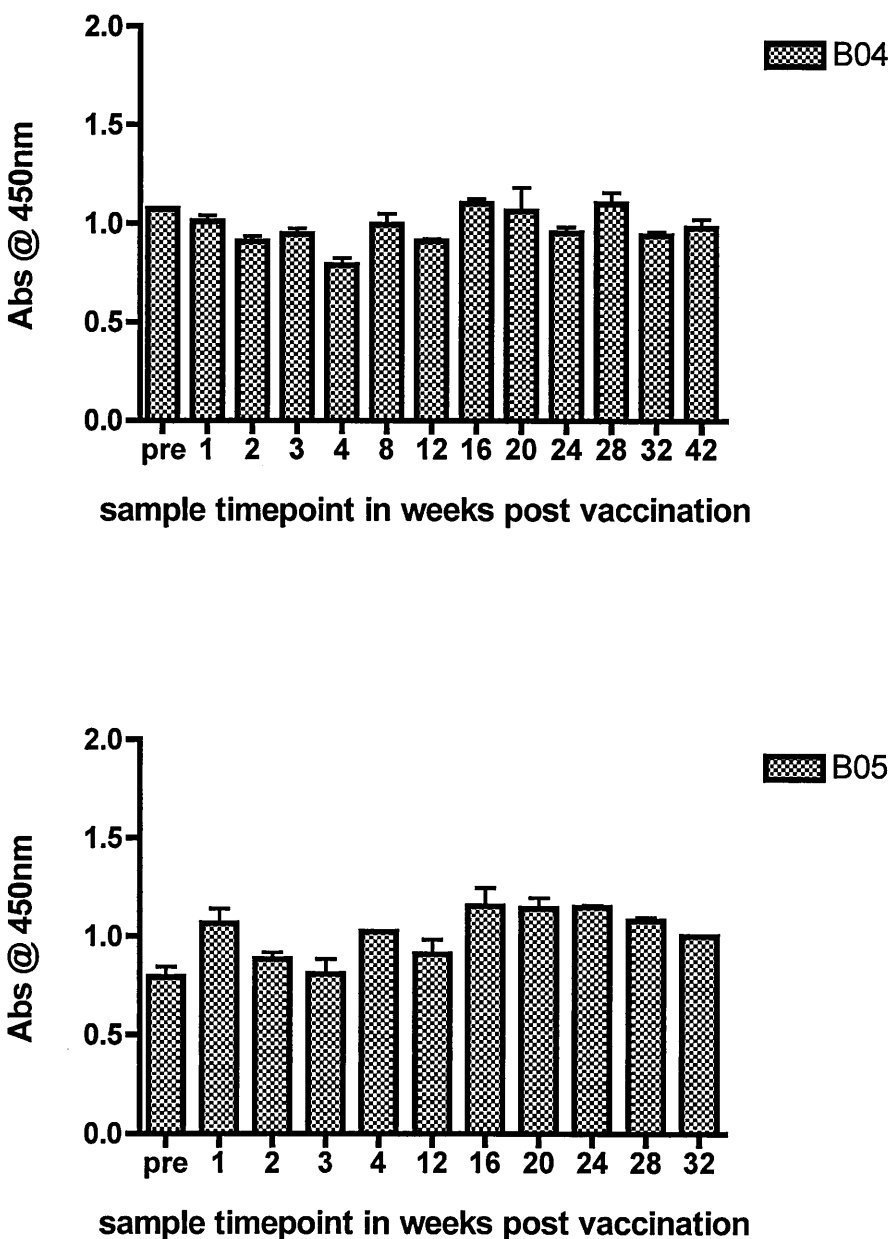


Figure 4.14 A-B Anti-tetanus activity in sequential patient samples. Several serum samples from selected patients were tested for the presence of specific IgG against tetanus by ELISA. The responses are shown as specific bound IgG. Each bar represents a 1:20 serum dilution, the timepoint of sampling in relation to the first vaccination is presented.

#### **4.4.7 Mutation of N linked glycosylation sites**

The patients showed no increase in IgG titre against cognate scFv-CaM during the course of the clinical trial. The presence of IgG reactivity against the cognate idiotype in patients M03, B04, B05 and S011 may be a true reflection of a pre-existing immune response against the malignant clone. Alternatively the positive reaction is due to antibodies against the glycan moieties on the recombinantly expressed scFv. Insect cells add less complex glycosylation than mammalian systems therefore the human sera may contain naturally occurring IgG antibodies against these glycan moieties. In order to test whether the human sera contains carbohydrate reactive antibodies it was necessary to mutate the asparagine residues N50 in B04 and N99 in B05. These were chosen for following reasons. Firstly, both had a single N-linked glycosylation site. Secondly, there is control donor serum that is strongly positive for both B04 and B05 scFv. Thirdly, since B05 did not bind Con A (Figure 3.17) and is therefore not expected to be glycosylated the alanine mutant will provide a suitable loss of function control. Finally, the B04 and B05 patient serum bind to their respective cognate scFvs.

##### **4.4.7.1 Site directed mutagenesis (SDM)**

B04 and B05 scFvs were mutated to incorporate an alanine residue in place of an asparagine residue at positions 50 and 99 respectively. The templates for the SDM were pMT/BiP/CaM-B04 and pMT/BiP/CaM-B05 (Appendix A). SDM was performed (Chpt. 2.2.1.17) using the primers in Table 4.3. The mutated sequence was confirmed by DNA sequencing (Chpt. 2.2.1.14). The mutated V<sub>H</sub>/V<sub>L</sub> gene cassettes of

B04 and B05 were sub-cloned into pMT/BiP/CaM; confirmation of correct gene insertion was by sequence analysis (Figure 4.15).

#### **4.4.7.2 Expression and purification of asparagine mutants**

pMT/BiP/CaM+B04-N50A and pMT/BiP/CaM+B05-N99A were expressed and purified from supernatant as before; the eluted fractions were analyzed on a 12% SDS-PAGE gel and stained with Coomassie blue (Figure 4.16). Interestingly the apparent molecular weight of the mutated B04-N50A is lower than the original B04 this is a good indicator that the protein has lost some or all of its glycosylation. The lack of glycosylation will make the protein traverse the gel faster. Purified B05-N99A appears to have the same molecular weight as B05, indicating that the mutation has not affected the glycosylation status of the protein. The concentration of protein in the purified fractions was determined by BCA assay (Chpt 2.2.2.10) with yields of 0.2 mg/ml and 0.7 mg/ml for B04-N50A and B05-N99A respectively.

|       | FR1   | CDR1           | FR2                                | CDR2                                   |
|-------|---|----------------|------------------------------------|--|
| V3-48 | 1.....10.....20.....30<br> <br>EVQLVESGGGLVQPGGSLRLSCAASGFTFS | .....<br>SYSMN | ....40.....<br> <br>WVRQAPGKGLEWVS | 50.....60.....<br> <br>YISSSSTIYYADSVK |
| B04   | ---V-----RW-----V-----S-N                                     | A-T-N          | -----R---I-                        | N-T--GF-KD-----                        |
| V3-23 | EVQLVESGGGLVQPGGSLRLSCAASGFTFS                                | SYAMS          | WVRQAPGKGLEWVS                     | AISGSGGSTYYADSVK                       |
| B05   | -ER-V-----F-----N   | -Y-M-          | -----                              | T-----VK-----                          |

|       | FR3  | CDR3             | FR4          |
|-------|--|------------------|--------------|
| V3-48 | ....70.....80.....90....<br> <br>GRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR | ....100.....<br> | 103<br>      |
| B04   | ---V-----G-KN---L---V-   | QNFEGGYVFDF      | WGQGTTLVTVSS |
| V3-23 | GRFTISRDNKNTLYLQMNSLRAEDTAVYYCAK                                   |                  |              |
| B05   | ---ST-----D-VNV-----V-----M-----                                   | NISMEI           | WGQGTTLVTVSS |

Figure 4.15 Amino acid sequences of the  $V_H$  genes encoding B04 and B05 are shown aligned to their closest germline genes 3-48 and 3-23 respectively. The mutated asparagine residue is in red. Identical amino acids are illustrated by dashes. Replacement mutations are indicated in uppercase. Numbering according to Kabat.

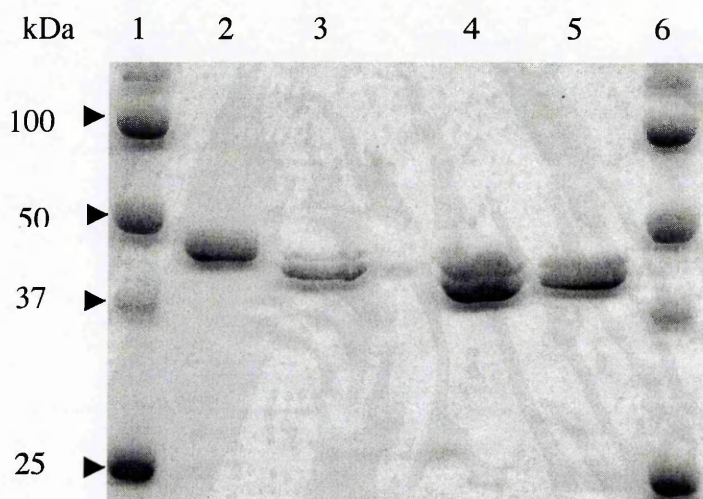


Figure 4.16 Coomassie stained SDS-PAGE gel of purified scFv-CaM. A stable S2 cell line expressing each scFv-CaM was scaled up in 200 ml of serum free medium before induction with 500  $\mu$ M  $\text{CuSO}_4$ . On day 5 post induction 200 ml of supernatant was harvested and supplemented with 1 mM  $\text{CaCl}_2$ . scFv -CaM was purified over a W7 column and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu$ l of each fraction analysed on a 12% SDS-PAGE gel and visualised with Coomassie blue. Lanes 1 and 6 – precision plus protein standard, Lane 2 - B04-CaM, Lane 3 - B04-N50A-CaM, Lane 4 - B05-CaM, Lane 5 – B05-N99A-CaM.

#### **4.4.7.3 Binding of human sera to B04-N50A and B05-N99A**

The purified alanine mutants of B04; -N50A and B05; -N99A were tested alongside the original purified samples of B04 and B05 to reassess the binding of the panel of eleven normal human serum to the scFvs. Results in table 4.8 show that all eleven serum samples bind to B04. However, the B04-N50A-CaM mutant reduces the binding of all serum samples to background (Figure 4.17). Only one serum sample showed reactivity against B05 (Table 4.8). This sample also showed reactivity against the asparagine mutant B05-N99A-CaM, the binding was slightly reduced but not to a significant degree (Figure 4.17).

The mutants were then used to reassess the binding of the cognate patient serum samples to the scFvs (Figure 4.18). Once again where the B04 patient samples exhibited activity against B04-CaM this was reduced to background levels against B04-N50A-CaM. There was no discernable difference between the pre- and post-vaccination samples. In contrast there is no difference in the binding of the B05 patient samples to B05-CaM compared to B05-N99A-CaM. Again there is no significant difference between the pre- and post-vaccination samples.

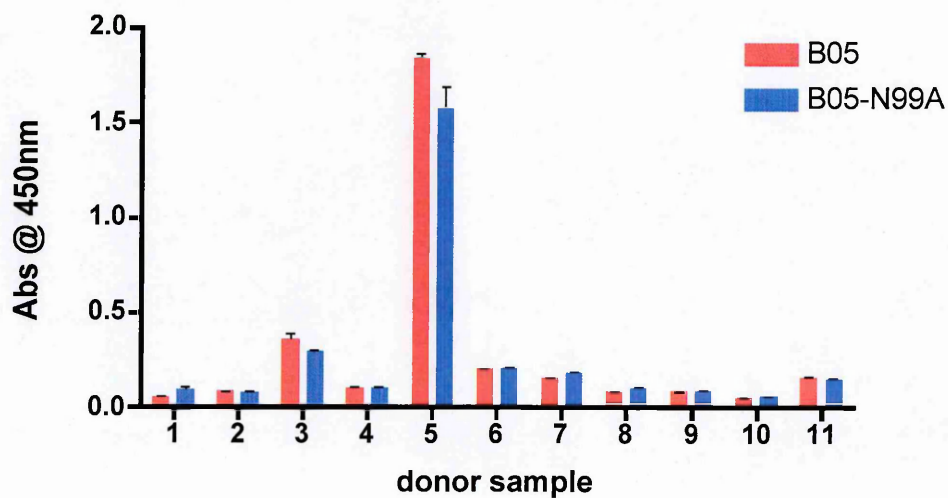
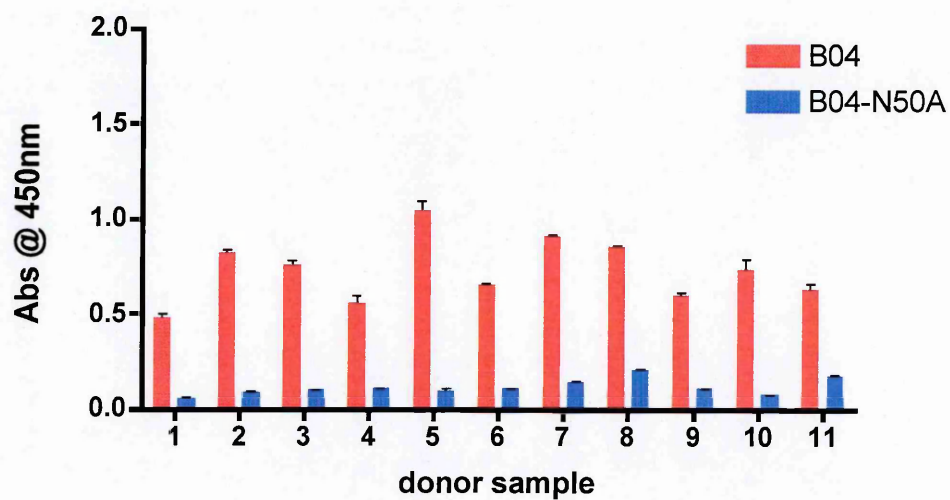


Figure 4.17 Comparison of anti-idiotype activity in normal human serum after removal of N-linked glycosylation on the recombinant scFv-CaM. Each bar represents a 1:20 serum dilution from an individual donor. The red bars correspond to the original scFv-CaM and the blue bars are the alanine mutants of the scFv-CaM. The results are shown as specific bound IgG.



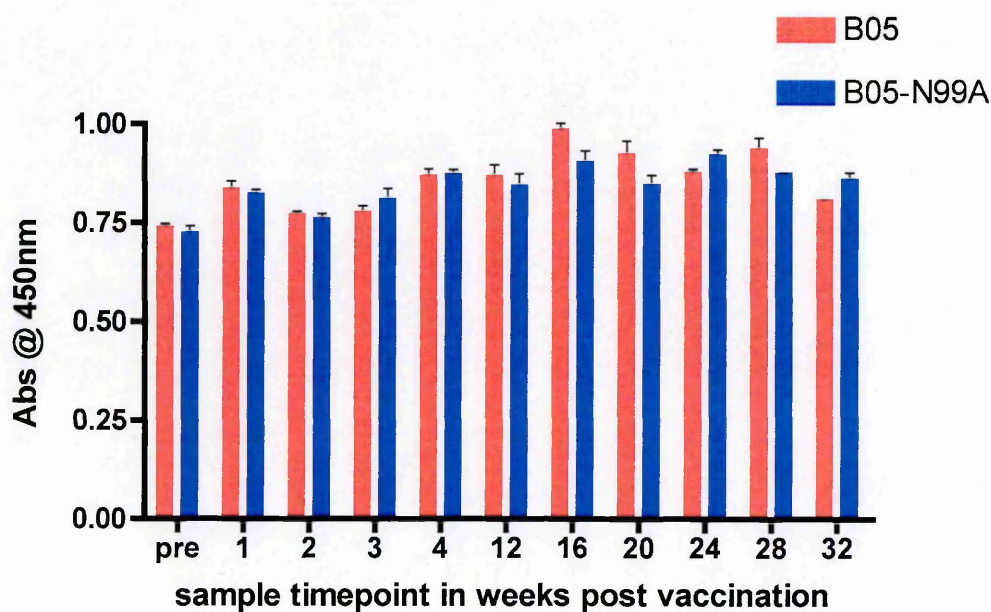
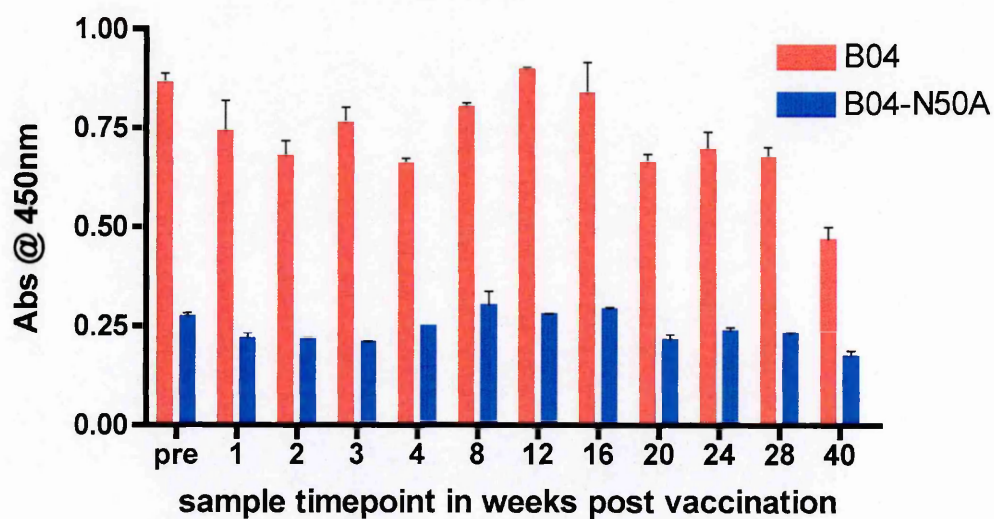


Figure 4.18 Comparison of anti-idiotypic activity against cognate scFv and unglycosylated cognate scFv in sequential patient samples. Each bar represents a 1:20 serum dilution. The red bars correspond to the original scFv-CaM and the blue bars are the alanine mutants of the scFv-CaM. The results are shown as specific bound IgG.

## 4.5 Discussion

The work described in this chapter is in two parts. Firstly, the details of the production and pre-release testing of the clinical grade DNA vaccines in the LIFTT clinical trial, CRC Code: 98/04, Protocol No: PH2/039. In the second part the efficacy of the vaccines to elicit a response against tetanus and against the cognate idiotypic is determined. For the latter the recombinant calmodulin fused scFvs expressed in S2 cells were used (Chapter 3).

The rearranged V genes derived from the malignant clone from each patient were assembled into a  $V_H/V_L$  gene cassette of a typical average length of 246 base pairs (range 237 – 263). The V gene cassettes were cloned into the plasmid pVAC2 by the collaborating clinical centres in Bournemouth, Manchester and Southampton. Each pVAC2-V gene construct was transferred to Cambridge for large-scale sterile preparation of the plasmid vaccines under conditions of Good Manufacturing Practice (GMP). At each stage of the process the V gene cassette was sequenced and aligned to the original sequence derived from the malignant clone. Any nucleotide differences in the sequences were re-analysed and a consensus was agreed between collaborators. The sequence of the 25 V gene cassettes is depicted in Appendix A and the clones carry all the classical hallmarks of rearranged V genes in lymphoma cells (Bahler & Levy, 1992; Zhu *et al.*, 1994; Zhu *et al.*, 2002). Most remarkably in 23 of the 25 clones putative N glycosylation sites had been introduced by somatic hypermutation or by the recombinatorial events (Zhu *et al.*, 2002).

A typical batch of patient-specific vaccine consisted of at least 15 ampoules of which five were used for pre-release testing. Vaccines were tested for sequence of the V gene cassette, intactness of the plasmid backbone by restriction enzyme digestion, DNA quantity (OD at 260 nm), DNA purity (OD 260/280 nm), sterility, and endotoxin level. The accepted range of 260/280 nm ratio was between 1.7 and 2.0 and values < 1.7 were failed because of protein contamination. Providing the quantitative and qualitative characteristics met the required standards the DNA vaccine was sterile filled into at least 12 single dose ampoules. Two ampoules each were used for endotoxin and sterility testing and one for sequencing and restriction enzyme digest.

A batch of vaccine was only released for clinical use if it had passed all the pre-release test criteria. The data shown in Table 4.5 only concerns the successful vaccines. Six batches of vaccine needed to be produced more than once for the following reasons. The first batch of S01 and the second batch of B03 did not yield adequate amounts of plasmid. The first batches of B03 and S013 failed sterility tests. Because of technical fermentation problems three unsuccessful production runs of S06 were completed. Finally, patients B01 and S05 relapsed before the vaccine could be administered. These patients re-achieved remission and were allowed re-entry into the trial, however, the initial batches of vaccine had exceeded their expiration dates resulting in the preparation of fresh vaccine.

In parallel to the clinical use of the vaccines, twenty-three were also administered to Dutch rabbits to determine the response against tetanus and against the cognate idiotypic.

All 23 vaccines were able to elicit an IgG response against tetanus toxoid. The responses varied between vaccines with four producing only a weak response after five immunisations. Poor responses against tetanus toxoid are exceptional when immunising with a standard protein vaccine. Seventeen of the 23 rabbits produced measurable IgG responses against the cognate idotype. Of the six remaining ones, the poor response to S012 and the lack of a response to M02 and S03 can be explained by poor expression of the scFv after plasmid delivery, a notion supported by the poor expression of the corresponding scFv-CaM in S2 cells. An alternative explanation of poor anti-Id responses is the relative low level of sequence diversity of the V domains with self. This could explain a poor immune response in humans but it is unlikely in rabbits as there is at least 40% sequence diversity between rabbit and human V domains. Interestingly S03 was one of the vaccines that also produced a weak response against tetanus indicating that there was relatively low expression of fragment C. Based on the experimental results we conclude that the majority of the vaccines are being transcribed and translated *in vivo* and that the recombinant fusion protein of scFv and FrC is available for antigen presentation. However, the magnitude of the response is rather blunted in a substantial subset of vaccines indicating that the amount of antigen produced is below the threshold to induce a robust B and T cell proliferation. Our observations are in line with those from others who have observed excellent immune responses in small laboratory mammals (mainly mice) whilst the magnitude of response was less impressive when testing larger animals.

The panel of recombinant scFvs and the immune rabbit serum generated by DNA vaccination with pVAC2-scFv provides a unique opportunity to ascertain whether the anti-idiotypic serum is universally cross-reactive or more specific in its binding. The

experiment was limited to include the 18 serum samples and recombinant scFv that in combination gave good to excellent IgG responses (Table 4.6). Of these 18 antisera and recombinant scFvs thirteen were specific in their binding to the cognate idiotypic and showed no significant binding to the non-cognate ones. The remaining five were shown to have cross-reactive profiles and were against so called public idiotypes (Roitt & Delves, 2001). Interestingly S05 and S09 are encoded by the same germline  $V_H$  and  $V_L$  genes and as expected the cross reactivity is high. The anti-S05 is equally reactive for both S05 and S09, conversely the anti-S09 although has a high reactivity for S05 it is not as high as with cognate. Obviously there are a number of shared epitopes between these two proteins, however the CDR3 of S09 is five amino acids longer than S05, which would offer an explanation for the titration curves of the cross-reactivity profiles (Figures 4.8 A and B). The implication is that a greater response to S09 is directed toward the CDR3, and therefore less of the total anti-Id IgG is against the common determinants shared between S05 and S09, which result in a decrease in the overall response to S05.

Patterns to the cross reactivity can be seen, generally scFvs derived from the same family of V genes or even the same germline gene are shown to be cross-reactive.

Control normal human serum samples were tested for the presence of anti-tetanus and anti-idiotypic. As expected all samples were strongly positive for anti-tetanus IgG as a direct result of the current immunisation program adopted for the control of tetanus infections (Figure 4.11). The patient samples were also strongly positive for anti-tetanus but no increase in activity was observed post vaccination (Figures 4.15). There are two possible explanations for the lack of increase in the anti-tetanus response post-vaccination. Either the fragment C of tetanus toxoid included in pVAC2 is incapable

of boosting the patients pre-existing response to tetanus because the fragment is not properly expressed or processed or the rise in antibody titre is too small to be detected by ELISA.

When testing normal human control serum samples for the presence of anti-idiotypic reactivity we were surprised to observe some robust positive reactions. All 11 serum samples were cross-reactive for at least three of the recombinant scFvs. Two of the 11 scFvs, B04 and M03, were recognised by all 11 serum samples. Conversely B05 and M02 were only recognised by one serum sample and there was no reactivity against M01. One possible explanation for the binding of IgG to idiotypic protein is the presence of a population of IgG antibodies against glycan moieties specifically conferred by S2 cells. However, close inspection of the primary amino acid sequence of the V domains for putative N-glycosylation sites and the Con A binding experiment (Table 3.1) showed that this was not the explanation for scFv clones B03, B05, M08 and B2. Clone B04 and B05 both contained the classic glycosylation sequons and Con A binding to the recombinant scFv B04, but not B05, was observed. Therefore the latter was used to test whether the binding of the IgG in sample 5 was dependent on the glycan moiety. The asparagine at positions 50 and 99 in clones B04 and B05 respectively were both mutated to alanine and both scFvs were expressed. The eleven normal human serum samples were tested against both mutants and no binding of IgG to scFv-B04-N50A was observed. However, serum sample 5 that was reactive against B05 remained reactive against the alanine mutant scFv-B05-N99A. There are two possible explanations for the observation with clone B04. Either, the serum samples did not contain specific anti-idiotypic against B04, instead they had naturally occurring IgG anti-carbohydrate activity directed against the glycans on the recombinant scFv,

or the asparagine itself was critical to IgG binding. The latter explanation is most likely as residue 50 resides in CDR2 and plays an important role in antibody binding and an asparagine to alanine mutation is severe. The binding of serum sample 5 to both B05 and B05-N99A strongly suggest that these potent IgG anti-idiotypic antibodies are present in the human serum samples.

A more suitable method to determine the contribution of the sugar residues in the binding assays would be to pre-treat the recombinant scFv with glycosylase enzymes.

The recombinant idiotypes were also used to investigate the sequential serum samples from ten patients who received their full six-vaccine course. We were not able to test the samples from the remaining 15 patients, as the clinical trial centre was reluctant to transfer samples. The anti-idiotypic activities observed in the ten patients are presented in Figures 4.13 A-E. There are two main types of reaction patterns, no reactivity above background (binding of IgG to D1D2) or significant binding but no increase of reactivity during the course of vaccination. Six (M01, M02, M04, M06, M08 and B03) were of the first type and four (M03, B04, B05 and S011) showed reactivity against cognate idiotypic. For patients B04 and B05 the assays were repeated with the alanine mutants of B04 and B05 respectively. The reactivity with B04-N50A was negligible strongly suggestive of the presence of anti-carbohydrate IgG activity in this patient. The B05 serum samples were equally reactive with B05 and B05-N99A. No definite conclusions can be drawn from this data set on the outcome of the clinical trial. Where a positive anti-idiotypic response was detected, it was not boosted after six vaccinations with pVAC2-scFv and none of the patients showed an increase in the titre of the anti-tetanus. Studies by our collaborators on T-cell mediated anti-Id responses were suggestive for some Id specific activity although the responses were weak in all

cases (Stevenson *et al.*, 2004). The conclusion of this DNA vaccination trial is that plasmid vaccination only is not sufficient to induce clinically effective responses against the idiotype in patients with FL. This observation is in line with other DNA vaccine trials in oncology or infectious diseases. Further improvements in DNA vaccine formulation will be required and particularly the results of priming with plasmid followed by boosting with Modified Vaccinia Ankara (MVA) seem to be a promising way forward. It is unlikely that this modification can be applied to FL patients, as the production of a patient specific MVA stock is laborious and expensive. Several groups have achieved promising results in phase II trials of patient-specific recombinant idiotype vaccines. Notably Genitope's phase I/II recombinant idiotype-keyhole limpet haemocyanin (KLH) co-administered with granulocyte-macrophage colony stimulating factor (GM-CSF) and Favril's phase II recombinant idiotype-KLH led to the initiation of phase III trials using the vaccine in patients after treatment. Currently three such idiotype-KLH GM-CSF phase III trials are operational in America, National Cancer Institute (NCI), Genitope and Favril. NCI and Genitope are treating patients using multi-agent chemotherapy, briefly NCI's regimen is prednisone, doxorubicin, cyclophosphamide and etoposide to best clinical response and the patients in the Genitope study receive eight cycles of cyclophosphamide, vincristine and prednisone. Patients that accomplish remission and are free from disease progression for a further six-month recovery period are entered for vaccination. Favril's patients are receiving a standard course of weekly Rituximab for four weeks prior to vaccination (reviewed in (Hurvitz & Timmerman, 2005)). In conclusion, vaccination against lymphoma idiotype appears to be a promising strategy, unfortunately the results from our phase I/II trial using DNA vaccination were disappointing.



## Chapter 5

### **Molecular characterisation of the variable domains of an $\alpha$ IIb $\beta$ 3 specific IgM $\kappa$ platelet cold agglutinin in a follicular lymphoma patient with profound thrombocytopenia**

#### **5.1 Introduction**

Classical pathogenic cold agglutinins (CA) are autoantibodies, usually of the IgM  $\kappa$  type that bind carbohydrate epitopes, most frequently I/i, on red blood cells (RBC) at low temperatures causing haemagglutination and haemolysis (reviewed in (Pruzanski & Katz, 1984; Roelcke, 1989)), a condition known as cold haemagglutinin disease (CHAD). This condition is frequently related with B-cell lymphoproliferative disorders and in most cases the CA is a monoclonal paraprotein. CA can also be associated with a variety of infections (McNicholl, 2000) particularly *Mycoplasma pneumoniae*.

Autoantibodies in chronic immune thrombocytopenia show reactivity at 37°C and are generally of the IgG class although IgA and IgM or combinations have also been reported (Kiefel *et al.*, 1996). Cold reactive platelet agglutinins are a rare phenomenon and only a few cases have been described, all of them IgM isotype using both  $\kappa$  and  $\lambda$  light chains where characterised. These antibodies, which recognise both EDTA dependant (van Vliet *et al.*, 1986; De Caterina *et al.*, 1993; Pujol *et al.*, 1998) and independent epitopes (Watkins & Shulman, 1970; Girmann *et al.*, 1984; van Vliet *et al.*, 1986; Cunningham & Brandt, 1992; Huss *et al.*, 1995; Muniz-Diaz *et al.*, 1995;

Schimmer *et al.*, 1998) were found associated with pseudothrombocytopenia. (Watkins & Shulman, 1970; van Vliet *et al.*, 1986; Cunningham & Brandt, 1992; De Caterina *et al.*, 1993; Schimmer *et al.*, 1998). None were associated with profound thrombocytopenia. Where the antigen was identified, binding to the glycoproteins  $\alpha$ IIb (GPIIb) (van Vliet *et al.*, 1986),  $\alpha$ IIb $\beta$ 3 (GPIIb/IIIa) (Schimmer *et al.*, 1998) or GP78 (De Caterina *et al.*, 1993) was demonstrated, however the involvement of carbohydrate moieties in the epitope was not determined.

All pathogenic anti-I/i CA studied to date use the heavy chain variable gene V<sub>H</sub>4-34 with minimal mutation from the germline (Potter *et al.*, 1993). The germline-encoded framework 1 and particularly the Ala-Val-Tyr sequence motif at position 23-25 have been shown to be critical to antibody specificity (Anderson *et al.*, 1996). Light chain use is also restricted with a marked preference for  $\kappa$  over  $\lambda$  and pathogenic anti-I almost always use V<sub>k</sub>3 light chains (Capra *et al.*, 1972; Gergely *et al.*, 1973; Silberstein *et al.*, 1991). Anti-I/i CA associated with infection are less restricted in their use of V genes (Smith *et al.*, 1995).

Immune thrombocytopenia is often seen in patients with B-cell lymphoproliferative disorders (Hedge *et al.*, 1985; Kuznetsov *et al.*, 1992; Lim & Ifthikharuddin, 1994). Rather than specific autoantibody being produced by the monoclonal malignant population itself, it is more likely that the inherent immunomodulated state in these diseases allows the emergence of autoreactive clones (Gupta *et al.*, 2000). In some cases the autoantibody is derived from the malignant clone and the thrombocytopenia can only be effectively corrected by controlling the malignant clone. In this chapter I present the molecular structure of the V domains of an IgM  $\kappa$  platelet CA in a patient with a low grade B-cell lymphoma and profound thrombocytopenia not responsive to 1<sup>st</sup>, 2<sup>nd</sup> and more advanced lines of treatment. Thrombocytopenia did however resolve

after a single treatment with a standard dose of Rituximab. The paraprotein derived from the malignant clone recognises the  $\alpha\text{IIb}\beta 3$  integrin, independent of platelet activation state and shows idiotypic overlap with our HPA-1a1a specific alloantibody B2.

## 5.2 Clinical Summary

The patient at the basis of this study concerns a 70-year-old female with a 10-year history of rheumatoid arthritis with low-grade B-cell non-Hodgkin's lymphoma and associated thrombocytopenia. A year later she was admitted to hospital with rectal bleeding accompanied by widespread petechiae, bruising, tongue and buccal mucosa bleeding and epistaxes. On admission she had haemoglobin of 7.4 g/dl (normal range 12-16.9 g/dl), a platelet count of  $12 \times 10^9/\text{l}$  (normal range  $150\text{--}450 \times 10^9/\text{l}$ ), a leucocyte count of  $19.4 \times 10^9/\text{l}$  (neutrophils 9.8, lymphocytes  $7.7 \times 10^9/\text{l}$ ) and her coagulation screen was normal. A bone marrow aspirate showed evidence of infiltration with low grade B-NHL and increased megakaryocytes. Immunophenotyping studies of the bone marrow revealed a  $\text{CD}20^+$ ,  $\text{FMC}7^+$ ,  $\text{CD}23^-$  and  $\text{bcl-2}$  negative B-cell population. Immunoglobulin gene rearrangement studies showed a monoclonal IgH rearrangement with a serum IgM paraprotein of 3.3 g/l and a trace of  $\kappa$  Bence Jones protein in the urine. Her chest X-ray and abdominal computed tomography (CT) scan were normal. She had a negative anti-nuclear antibody and rheumatoid factor was elevated at 107 iu/ml.

Her bleeding was treated with platelet transfusion, intravenous immunoglobulin and prednisolone 1mg/kg/day. There was no response and she then went on to receive azathioprine with no response, vincristine 1.5 mg weekly for three weeks again with

no response and further intravenous immunoglobulin. During this time her platelet count fluctuated between 4 and  $14 \times 10^9/l$ .

Subsequent treatment for her refractory immune thrombocytopenia (ITP) included tranexamic acid, plasma exchange and further intravenous immunoglobulin, all with no response. When platelet antibody investigations revealed the presence of a potent IgM  $\kappa$  cold agglutinin a link between the intractable thrombocytopenia and the malignant clone was suggested and she received Rituximab (anti-CD20 monoclonal antibody, Mabthera) at a dose of  $375 \text{ mg/m}^2$  as an intravenous infusion weekly for four weeks. On Day +6 of the first dose of Rituximab her melaena had stopped, and on Day +20 her platelet count had risen to  $17 \times 10^9/l$  rising further to  $50 \times 10^9/l$  and a bone marrow examination on Day +41 was normal with complete remission of her B-NHL on morphological, immunophenotypic, and gene rearrangement analyses. Further routine investigations performed by the platelet laboratory at the National Blood Service Cambridge revealed that  $\alpha\text{IIb}\beta 3$  was the likely antigen recognised by the monoclonal IgM (personal communication with Graham Smith).

## **5.3 Experimental Design**

### **5.3.1 Determination of platelet antigen**

The serum from the patient was used in the platelet immunofluorescence test (PIFT) (Chpt. 2.2.6). HPA typed panel platelets and platelets from a Glanzmann's Thrombasthenia (GT) patient were used and the assay was performed at a variety of temperatures.

### **5.3.2 Isolation of V genes**

To establish whether the dominant clone of the Follicular Lymphoma was responsible for the patients' thrombocytopenia the rearranged V genes were rescued from a bone marrow biopsy and an scFv was constructed.

#### **5.3.2.1 Amplification of V genes**

Total RNA was isolated from a bone marrow biopsy taken from the patient (Chpt 2.2.1.1) and first strand cDNA was synthesised (Chpt 2.2.1.5). Rearranged V<sub>H</sub> and V<sub>k</sub> genes were amplified by PCR using signal peptide primers specific for each gene family in combination with C<sub>γ</sub>, C<sub>μ</sub> or C<sub>κ</sub> specific primers (Table 5.1) (Dohmen *et al.*, 2005). PCR products were cloned into pCR2.1 (Invitrogen) (Chpt 2.2.1.7) and sequenced (Chpt 2.2.1.14). The V-base directory (Cook & Tomlinson, 1995) was used to identify the germline genes prior to amplification with FR1/J region primers to

append restriction sites (Table 5.1) without modifying the amino acid sequence. PCR products were cloned into pCR2.1 as above and sequenced. Clones with unmodified FR1 sequences (some primers are degenerate) were excised using the appended restriction sites and transferred to the phage display vector pHenIX (Finnern *et al.*, 1997) to generate an scFv cassette.

#### **5.3.2.2 Removal of IgG surface positive B-cells from bone marrow biopsy**

IgG membrane positive B-cells were removed from a bone marrow biopsy sample by antibody mediated magnetic separation. Purification was carried out according to the manufacturer's protocol; briefly, 1 ml BioMag (Perseptive Biosystems, Framingham, USA) goat anti-mouse IgG beads were saturated (8 mg) with mouse monoclonal anti-human IgG (Dako Cytomation) and then added to  $1 \times 10^6$  cells. After incubation at RT for 1 hour, IgG<sup>+</sup> cells were removed and the remaining cells washed with PBS.

#### **5.3.3 Anti-idiotypic antibodies by DNA vaccination**

V<sub>H</sub>V<sub>L</sub> gene cassettes were excised from pHenIX as Sfi I/Not I fragments and cloned into the vaccination plasmid pVAC2. Plasmids were maintained in *E. coli* strain DH5 $\alpha$ . DNA for vaccination was prepared using a Qiagen Plasmid Giga Kit (Qiagen) (Chpt 2.2.1.12). Dutch rabbits were immunised three times intramuscularly with 200  $\mu$ g of plasmid at two weekly intervals and a test bleed was taken. Animals that failed to respond were given two additional immunisations (Chpt 2.2.3.1).

#### **5.3.4 Purification of whole IgM from patient serum**

IgM was purified from patient serum using goat anti-human IgM agarose. Briefly, 0.5 ml of goat anti-human IgM agarose (Sigma) was added to a Biorad poly-prep chromatography column. The agarose was washed with 20 ml of PBS before 10 ml of patient serum was passed over the column. The column was washed with 20 ml of PBS and the bound IgM was eluted in 1 ml fractions with 0.1 M glycine-HCl pH 2.5. Fractions containing protein were dialysed in PBS and the IgM concentration determined by BCA assay (Chpt 2.2.2.10), all steps were performed at 4°C.

| Primer Name                | Sequence 5'-3                             |
|----------------------------|---|
| C $\gamma$                 | GTCCACCTTGGTGTGCTGGGCTT                   |
| C $\mu$                    | TGGAAGAGGCACGTTCTTTTCTTT                  |
| C $\kappa$                 | AGACTCTCCCCTGTTGAAGCTCTT                  |
| V <sub>H</sub> 1a/7 signal | ATGGACTGGACCTGGAGGRTC                     |
| V <sub>H</sub> 1b signal   | ATGGACTGGACCTGGAGMATC                     |
| V <sub>H</sub> 2 signal    | ATGGACACACTTTGCTMCACRC                    |
| V <sub>H</sub> 3 signal    | ATGGAGTTTGGGCTGAGCTGG                     |
| V <sub>H</sub> 4 signal    | ATGAAACACCTGTGGTTCTTCC                    |
| V <sub>H</sub> 5 signal    | ATGGGGTCAACCGCCATCCT                      |
| V <sub>H</sub> 6 signal    | ATGTCTGTCTCCTTCCTCATCT                    |
| V $\kappa$ 1 signal        | ATGAGGGTCCCYGCTCAGC                       |
| V $\kappa$ 2 signal        | ATGAGGCTCCCTGCTCAGC                       |
| V $\kappa$ 3a signal       | ATGGAAACCCAGCGCAGC                        |
| V $\kappa$ 3b signal       | ATGGAGGCCCCAGCACAGC                       |
| V $\kappa$ 4 signal        | ATGGTGTTCAGACCCAGG                        |
| V $\kappa$ 5 signal        | ATGGGGTCCCAGGTTCAAC                       |
| V $\kappa$ 6a signal       | ATGTTGCCATCACAACTCATTG                    |
| V $\kappa$ 6a signal       | ATGGTGTCCCCGTTGCAATTC                     |
| V <sub>H</sub> 1a Sfi I    | AACTGCGGCCAGCCGGCCATGGCCCAGGTCAGCTGGTGCAG |
| C0 V <sub>H</sub> 1b Sfi I | AACTGGGCCAGCCGGCCATGGCCCATGTGCAGCTGGTTCAG |
| V $\kappa$ 3a ApaL I       | CACAGTGCACTCGAAATTGTGTTGACRCAG            |
| J <sub>H</sub> 4-5 Sal I   | CTCGTGTGACACGGTGACCAGGGTTCC               |
| J $\kappa$ 5 Not I         | ACTTGCGGCCGCACGTTTAATCTCCAGTCGTGTC        |

Table 5.1 Nucleotide sequences of the signal peptide specific primers for amplification of the rearranged V genes and FR1 and J region primers to append SfiI/SalI and ApaLI/NotI restriction sites.



## 5.4 Results

### 5.4.1 PAIg

Direct and indirect PIFT were performed to determine the presence of PAIg. The direct PIFT when performed at 20°C was strongly positive for IgM. The patient's plasma was tested by indirect PIFT at 20°C and gave a strong positive reaction with anti-IgM and anti- $\kappa$  reagents only (Figure 5.1); the strength of reaction was independent of the HPA genotype of the panel cells (data not shown). Testing over a range from 4-37°C showed the binding of the IgM antibody to be strongly temperature dependent with a clearly positive reaction up to 25°C, weak reactions at 30 °C and negative at 37°C (Figure 5.2). The binding of the IgM in the presence and absence of EDTA (data not shown) indicated that the antibody does not recognise an EDTA dependent epitope. The temperature dependence of the platelet IgM is suggestive of a low affinity antibody. Determining the specificity of a low affinity antibody is cumbersome. Therefore I investigated whether the patients IgM reacted with platelets from a patient with Glanzmann's Thrombasthenia (GT). GT is a serious, but rare, autosomal recessive disorder, characterised by longer bleeding times and absence of platelet aggregation. GT platelets lack the  $\alpha$ Ib $\beta$ 3 complex.

PIFT was performed as before, RFGP56 (a murine monoclonal anti- $\alpha$ Ib $\beta$ 3 which blocks platelet aggregation and fibrinogen binding to  $\alpha$ Ib $\beta$ 3) was used as a control. Both patient IgM (at 4°C) and RFGP56 (at RT) bound to normal platelets but neither bound the GT platelets (Figure 5.3).

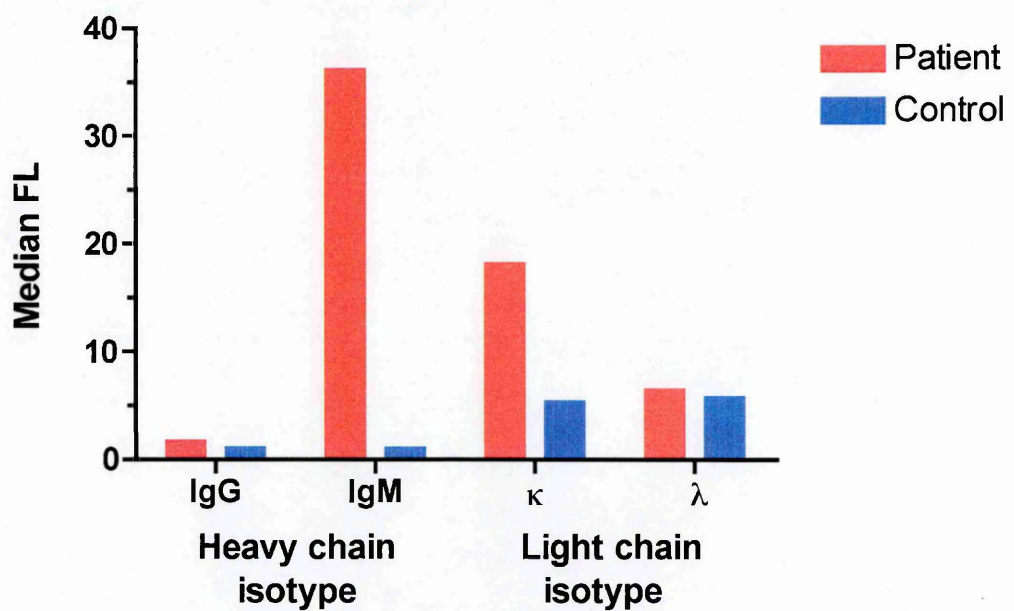


Figure 5.1 Immunofluorescence test on patient serum. Donor platelets were reacted with the patient sample (or an inert control) and binding of immunoglobulin was detected with isotype and light chain specific FITC labelled conjugates at 20°C. Bound FITC was measured by flow cytometry and results expressed as median fluorescence intensity (MFI) of 10,000 events. Reproduced by kind permission K. Campbell, Platelet Immunology Laboratory, National Blood Service, Cambridge.

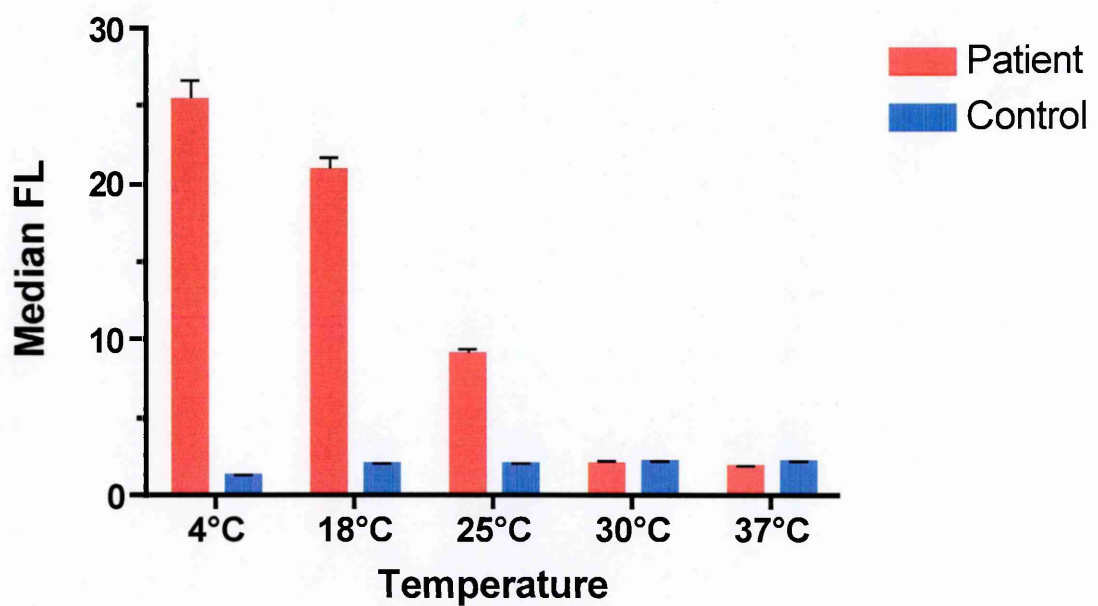


Figure 5.2 Temperature dependence of patient IgM. Donor platelets were reacted with the patient serum (red bars) or an inert control serum (blue bars) at increasing temperatures. Binding of immunoglobulin was detected with anti-human IgM FITC labelled conjugate. Bound FITC was measured by flow cytometry and results expressed as median fluorescence intensity (MFI) of 10,000 events.

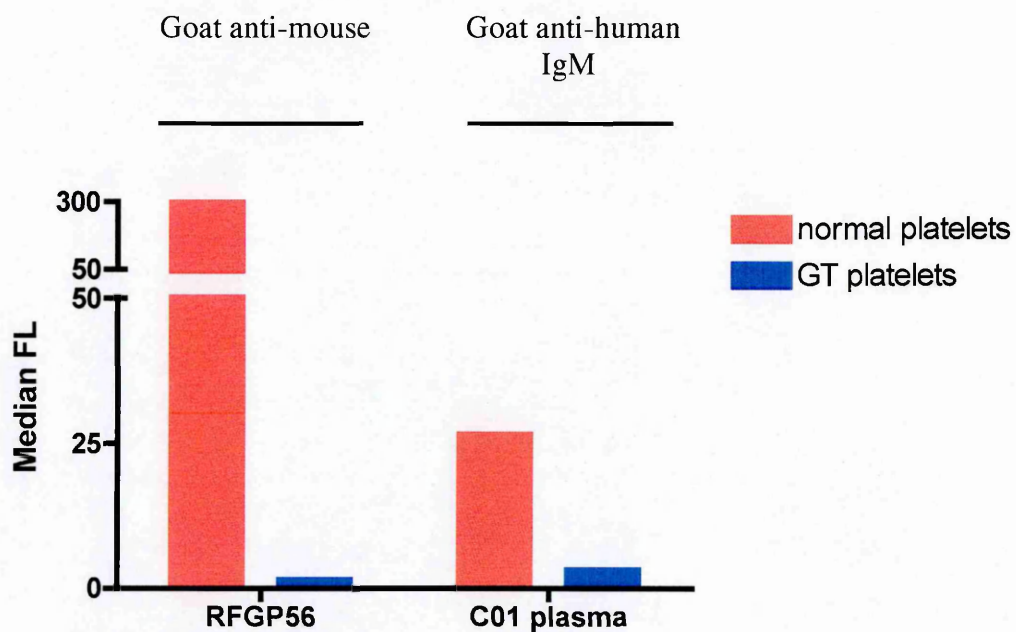


Figure 5.3 identification of the platelet antigen as  $\alpha$ IIb $\beta$ 3. Mouse monoclonal RFGP56 (anti- $\alpha$ IIb $\beta$ 3) (left) and patient serum (right) was reacted with donor platelets (red bars) or Glanzmann's Thrombasthenia (GT) platelets (blue bars). Binding of immunoglobulin was detected with the corresponding FITC labelled conjugate (as shown). Bound FITC was measured by flow cytometry and results expressed as median fluorescence intensity (MFI) of 10,000 events.

### 5.4.2 Isolation of V genes encoding IgM secreted by malignant clone

First strand cDNA synthesis of the extracted RNA from the bone marrow biopsy was used in RT-PCR to isolate the dominant lymphoma variable genes. Primers against the constant region of IgM ( $C_\mu$ ) and IgG ( $C_\gamma$ ) were paired with family specific variable heavy chain primers  $V_H$  1-6 (Table 5.1). Primers against the constant region of the kappa light chain ( $C_\kappa$ ) were paired with family specific variable light chain primers  $V_\kappa$  1-6 (Table 5.1).

Products of the expected size (~700 bp) were amplified from the cDNA using all combinations of  $V_H$  primers and  $C_\gamma$  (Figure 5.4 A), only with the  $V_H$ 1 primers when priming with  $C_\mu$  (Figure 5.4 B) and all  $V_\kappa$  primers and  $C_\kappa$  combinations (Figure 5.4 C). To increase the chance of obtaining the appropriate  $V_\kappa$  gene RT-PCR was also performed with the RNA obtained from the IgG<sup>+</sup> depleted bone marrow biopsy. RT-PCR amplified products of ~700bp using combinations of  $V_H$ 1a and  $V_H$ 1b primers with  $C_\mu$  (Figure 5.5 A) and  $V_\kappa$ 1 and  $V_\kappa$ 3 primers with  $C_\kappa$  (Figure 5.5 B). No products were amplified using any combination of  $V_H$  primers and  $C_\gamma$  (data not shown).

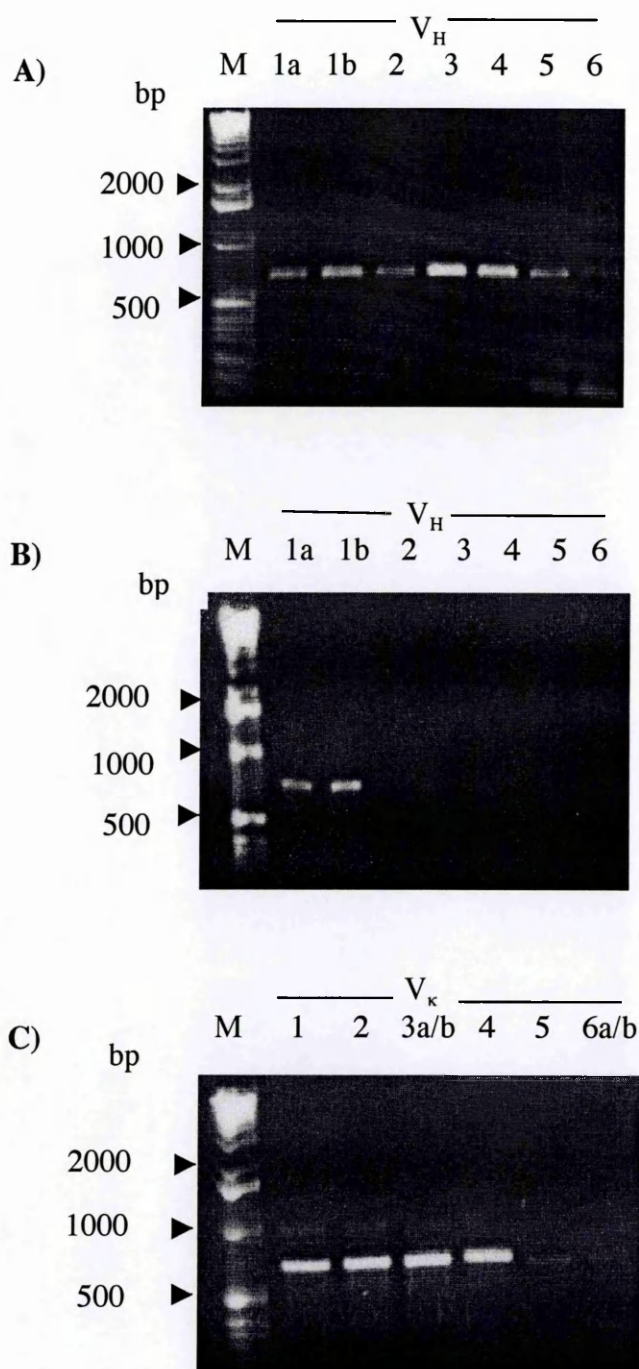


Figure 5.4 Rescue of rearranged V<sub>H</sub> and V<sub>K</sub> genes from a bone marrow aspirate by RT-PCR based amplification. V gene family specific signal peptide primers (indicated) were combined with the human immunoglobulin constant region primers. 5 µl of PCR product was analysed on a 1% agarose gel. A) IgG constant region primer (C<sub>γ</sub>), B) IgM constant region primer (C<sub>μ</sub>) and C) kappa light chain constant region primer (C<sub>κ</sub>). M – 1 kb DNA ladder (Gibco BRL).

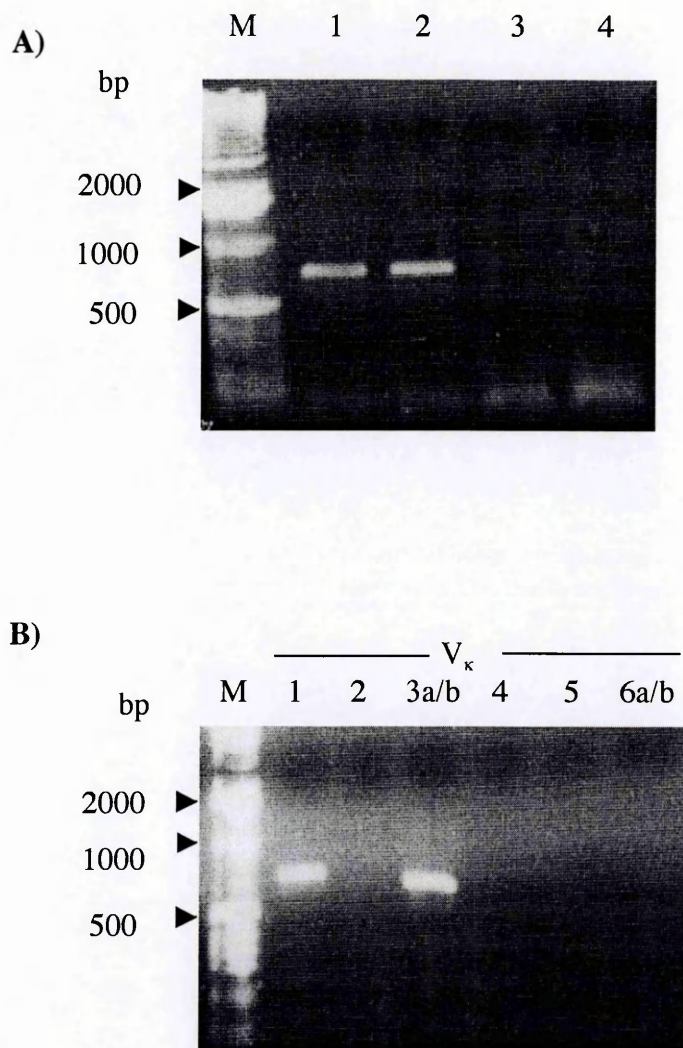


Figure 5.5 Rescue of rearranged  $V_H$  and  $V_\kappa$  genes from the IgG+ B-cell depleted RNA sample. 5  $\mu$ l of PCR product was analysed on a 1% agarose gel. A) IgM constant region primer ( $C_\mu$ ) with VH1a and VH1b specific primers. Lanes 1 and 3 – VH1a, lanes 2 and 4 – VH1b, lanes 3 and 4 are negative controls and contain no DNA. B) kappa light chain constant region primer ( $C_\kappa$ ) with  $V_\kappa$  1-6 specific primers (indicated). M – 1 kb DNA ladder (Gibco BRL).

The PCR products amplified with V<sub>H</sub>1/ C<sub>μ</sub> and V<sub>k</sub>1/ C<sub>κ</sub> and V<sub>κ</sub>3/ C<sub>κ</sub> from the IgG<sup>+</sup> B cell depleted samples were inserted into the pCR2.1 vector and 20 random clones sequenced. Two dominant V<sub>H</sub> gene rearrangements were identified (C01V<sub>H</sub>a and C01V<sub>H</sub>b (Figure 5.6 A)), with very similar sequences and identical Complementarity Determining Regions (CDR) 3 loops encoded by the D<sub>H</sub>5-24 and J<sub>H</sub>4-02 gene segments. Both are derived from the V<sub>H</sub>1-02 germline gene having 96.6/97.3% and 96.6/93.9 % homology with germline at nucleotide/amino acid level respectively. Nucleotide differences from the germline V<sub>H</sub>1-02 and J<sub>H</sub>4-02 gene elements encode four amino acid replacements located in FR3 with the exception of the A>T transversion in CDR3 leading to replacement of tyrosine (Tyr) with phenylalanine (Phe). A further two common silent mutations were found in the V<sub>H</sub> and J<sub>H</sub> segments. The C01V<sub>H</sub>a domains had 4/2 unique replacement/silent mutations from V<sub>H</sub>1-02 whereas the C01V<sub>H</sub>b had 5/2 unique replacement/silent ones. Sequencing of the V<sub>κ</sub>1/C<sub>κ</sub> PCR products revealed diverse unrelated clones, whereas a single dominant clone was identified from the V<sub>κ</sub>3/C<sub>κ</sub> PCR products (Figure 5.6 B) that uses a V<sub>κ</sub> gene with 99.3/97.9% homology (nucleotide/amino acid) to the V<sub>κ</sub>3-20 germline gene in combination with the J<sub>κ</sub>5 gene.



|                     | FR1   | CDR1                       | FR2                                 | CDR2                                     |
|---------------------|---|----------------------------|-------------------------------------|--|
| V <sub>H</sub> 1-02 | 1.....10.....20.....30<br> <br>QVQLVQSGAEVKKPGASVKVSKASGYTFT          | .....<br>GYMH              | .....40.....<br> <br>WVRQAPGQGLEWMG | 50.....60.....<br> <br>WINPNSGGTNYAQKFQG |
| C01VHa              | -V-----   | D--N                       | -----                               | -----K-----                              |
| C01VHb              | HV-----   | g--I-                      | -----                               | -----D-----                              |
|                     |   |                            | CDR3                                | FR4                                      |
|                     |   |                            |                                     |  |
| V <sub>H</sub> 1-02 | .....70.....80.....90.....<br> <br>RVTMTRDTSIS TAYMELSR LRSDDTAVYYCAR | ...100.....<br> <br>VEMATI | YFDY                                | WGQGLVTVSS                               |
| C01VHa              | W-----V--e-----G-----T  | DQ---SRAGVSEG--F           | -----                               | -----                                    |
| C01VHb              | W-----T-----M--G-----T  | DQ---SRAGVSEG--F           | -----                               | -----                                    |

Figure 5.6 A Amino acid sequences of the VH genes encoding C01VHa and C01VHb are shown aligned to the closest germ-line gene V1-02. Identical amino acids are illustrated by dashes. Amino acids encoded by silent mutations are indicated in lowercase, replacement mutations are indicated in uppercase. Numbering according to Kabat. Genbank accession numbers AY957509 and AY957510.

|        | FR1   | CDR1                         | FR2                                   | CDR2                    |
|--------|---|------------------------------|---------------------------------------|-------------------------|
| Vk3-20 | 1.....10.....20..<br> <br>EIVLTQSPGTLISLSPGERATLSC              | .....30..<br> <br>RASQSVSSSY | .....40.....<br> <br>LAWYQQKPGQAPRLLI | 50.....<br> <br>YGASSRA |
| C01Vk  | -----   | -----                        | -----                                 | --T----                 |
|        |   |                              |                                       |                         |
|        | FR3   | CDR3                         | FR4                                   |                         |
| Vk3-20 | ...60.....70.....80.....<br> <br>TGIPDRFSGSGGTFTLTISRLEPEDFAVYY | .90.....<br> <br>CQQYGSPP    | ..100.....108<br> <br>TFGQGTREIK      |                         |
| C01Vk  | -----   | -----A-----                  | -----                                 |                         |

Figure 5.6 B Amino acid sequence of the VL gene encoding C01Vk is shown aligned to the closest germ-line gene Vk3-20. Identical amino acids are illustrated by dashes. Replacement mutations are indicated in uppercase. Numbering according to Kabat. Genbank accession number AY957511.

### 5.4.3 Construction of scFvs C01a and C01b

Two scFvs were constructed using the two dominant V<sub>H</sub> sequences and the one V<sub>κ</sub>. The V<sub>κ</sub>3 gene was removed from the pCR2.1 plasmid by restriction enzyme digest (Chpt 2.2.1.15) with ApaLI and NotI (~ 300 bp band) (Figure 5.7 A) and cloned into pHenIX cut with ApaLI and NotI (~4 kb band) (Figure 5.7 A). V<sub>κ</sub>3 was ligated into pHenIX and transformed into electrocompetent TOP 10F. Plasmid minipreps of pHenIX- V<sub>κ</sub>3 were prepared and recombinants identified by digestion with ApaLI and NotI (Figure 5.7 B). The ~300 bp band indicates that the V<sub>κ</sub>3 has been successfully cloned into 5 of the 7 plasmids tested (Figure 5.7 B).

The C0V<sub>H</sub>1a and C0V<sub>H</sub>1b genes were removed from the pCR2.1 plasmid by restriction enzyme digest with SfiI and SalI, the 400 bp band was gel purified (Figure 5.7 C) and each was cloned into pHenIX- V<sub>κ</sub>3 as above. Plasmid minipreps were prepared and confirmation of V gene insertion was by sequence analysis (Chpt 2.2.1.14) (Appendix A). The resulting scFvs were named C01a and C01b.

### 5.4.4 Expression and purification of scFvs C01a and C01b

scFvs C01a and C01b were removed from the pHenIX plasmid by restriction enzyme digest with NcoI and NotI and subcloned into the pMT/BiP/CaM expression plasmid. Recombinants were identified by sequencing. Stable S2 cell lines of pMT/BiP/CaM-C01a and pM/BiP/CaM-VC01b were produced as before (Chpt 3.3.4). The level of expressed scFv in the supernatant 5 days post induction was determined by Western blotting (Figure 5.8 A) and scFv-C01a-CaM and C01b-CaM were grown up in 200 ml

of serum free media and expression induced for 5 days. The supernatants were harvested and purified over W7 columns; 10  $\mu$ l of the eluted fractions were analyzed on a 12% SDS-PAGE gel and stained with Coomassie blue (Figure 5.8 B). The concentration of protein in the purified fractions was determined by BCA assay (Chpt 2.2.2.10) with yields of 0.4 mg/ml and 0.7 mg/ml for C01a and C01b respectively.

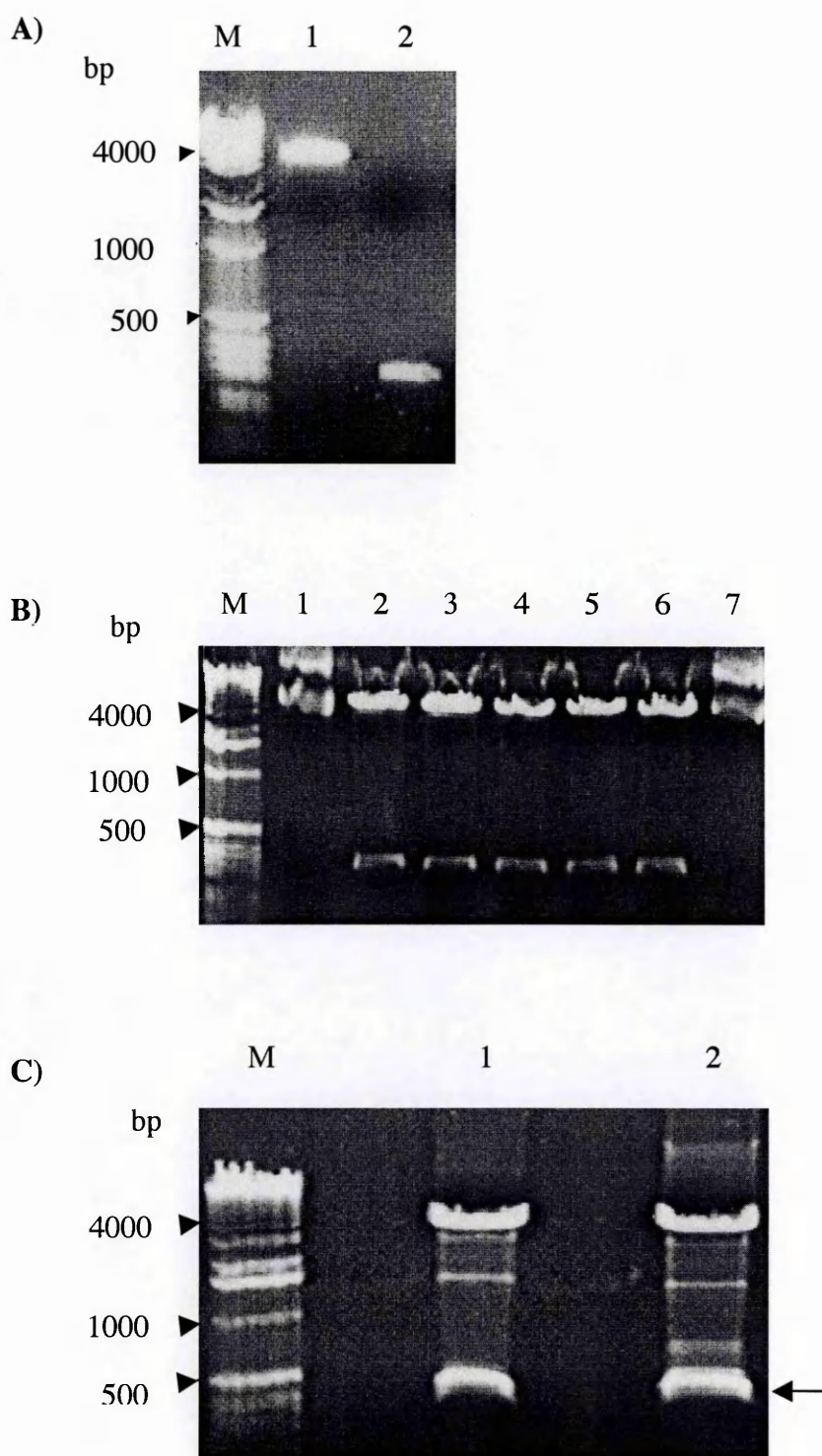


Figure 5.7 Restriction enzyme digests of plasmids for the construction of scFv. A) Gel extracted ApaLI/NotI digests of lane 1 pHenIX (~4 kb band), lane 2 Vk3 (~300 bp band) from pCR2.1. B) Confirmation of Vk3 insertion into pHenIX by ApaLI/NotI digest lanes 2-6 and no insert lanes 1 and 7 (absence of 300 bp band). C) SfiI/SalI digests of pCR2.1-VC01a, lane 1 and pCR2.1-VC01b, lane 2. The ~400bp band marked by the arrow is the  $V_H$  gene and was excised from the gel and purified. M – 1 Kb DNA ladder.

A)

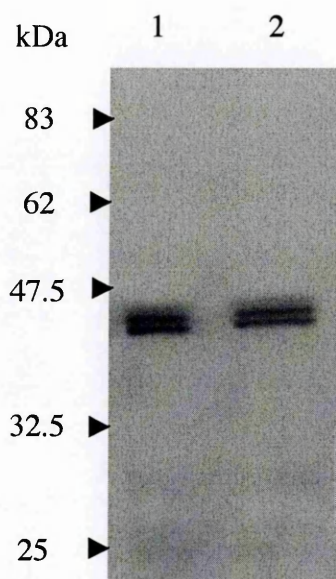


Figure 5.8 A Detection of C01a-CaM and C01b-CaM by immunoblot. Stable S2 cell lines expressing C01a-CaM and C01b-CaM were scaled up in 200 ml serum free media before induction with 500  $\mu$ M  $\text{CuSO}_4$ . A 1 ml sample was taken at day 5-post induction and 10  $\mu$ l of cleared supernatant analysed on a 12% SDS-PAGE gel. CaM tagged proteins were detected with peroxidase labelled N9A and visualised by enhanced chemiluminescence. Lane 1 C01a-CaM and lane 2 C01b-CaM.

B)

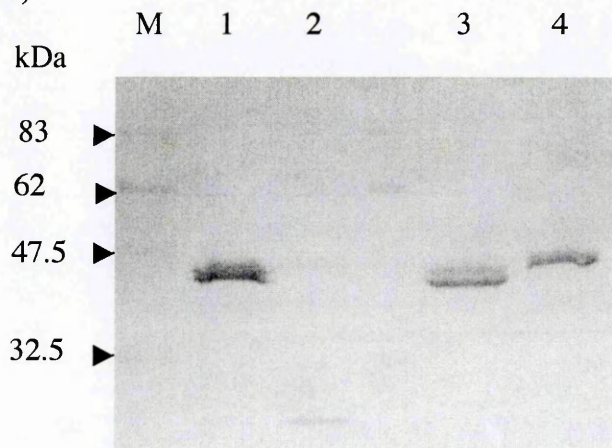


Figure 5.8 B Coomassie stained SDS-PAGE gel of purified scFvs C01a and C01b-CaM. S2 cells were treated as for Figure 5.6A. On day 5-post induction 200 ml of supernatant was harvested and supplemented with 1 mM  $\text{CaCl}_2$ . scFvs were purified over a W7 column and eluted in 1 ml fractions with TBS containing 10 mM EDTA. 10  $\mu$ l of each fraction was analysed on a 12% SDS-PAGE gel and visualised with Coomassie blue (Chpt 2.2.2.8.1). Lanes 1 and 2 C01a-CaM fractions 2 and 3, lanes 3 and 4 C01b fractions 2 and 3. M Broad range molecular weight marker.

#### 5.4.5 Production of anti-idiotypic sera

Both V gene cassettes were subcloned into the DNA vaccination vector pVAC2.

Dutch rabbits were immunised as before and bleeds were taken at days 0 and 35. IgG responses against tetanus and idiotype was measured by ELISA (Figure 5.9 and 5.10).

Anti-C01a bound to TEFT (Figure 5.9 A) and its cognate CaM tagged scFv derived from the patient, but not an irrelevant CaM tagged protein (D1D2) (Figure 5.10 A).

The rabbit immunised with pVAC2-C01b failed to make an anti-tetanus (Figure 5.9 A) or anti-idiotypic response after 3 immunisations (Figure 5.10 A). The rabbit was boosted twice with 200 µg of pVAC2-C01b at days 42 and 49 and a third bleed was taken at day 56. The anti-tetanus and anti-idiotypic responses were measured again (Figure 5.9 B and 5.10 B); both anti-tetanus and anti-idiotypic responses were produced although not as high a titre as the anti-C01a.

#### 5.4.6 Cross reactivity of anti-idiotypic sera

The antisera were also screened against the panel of twenty-one scFvs derived from patients with follicular lymphoma (Chapter 4) and scFv-B2 (Chapter 3). 5/21

lymphoma scFvs and B2 were positive for the C01 idiotype (>20% of cognate signal at 1/20 dilution of sera) with a range of cross-reactivity (Figure 5.11). scFv-B2 is

derived from the same germline V<sub>H</sub> gene as C01VHa/b and had almost identical

reactivity to that of the cognate scFv. scFv-B04 which is encoded by a member of the

V<sub>H</sub>3 gene family but has a kappa light chain derived from the same germline gene also demonstrated strong cross-reactivity as did S011 for which both heavy and light chains are encoded by members of different V gene families. C01 also shares some idiotypic

determinants with three other lymphoma scFvs (S04, S06 and M08) tested that are encoded by diverse V genes. The homology of C01V<sub>H</sub>a and C01V<sub>H</sub>b to these genes is listed in Table 5.2. Anti-idiotypic serum raised against B2 was strongly cross-reactive with C01a and C01b (data not shown).



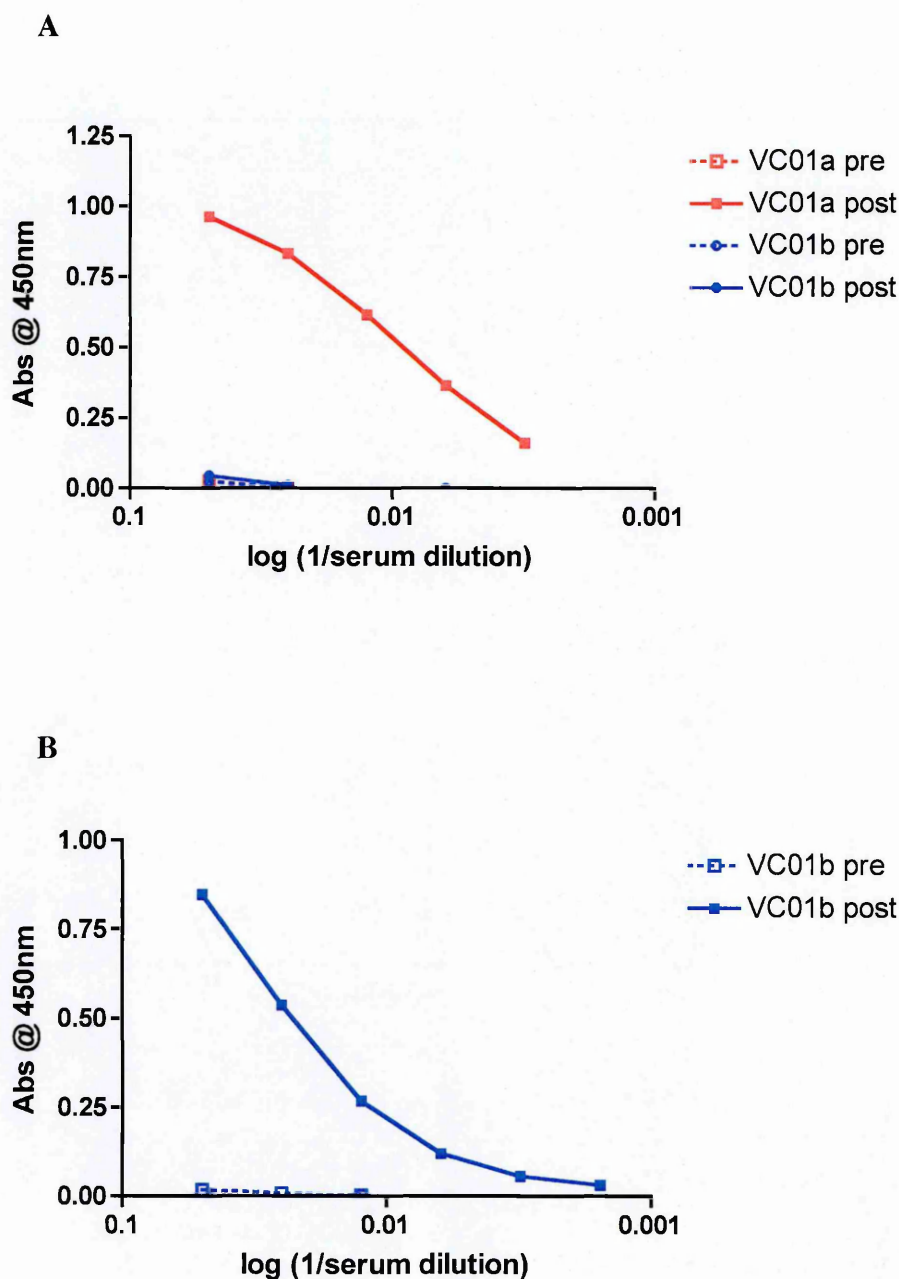


Figure 5.9 Anti-tetanus responses in Dutch rabbits immunised with pVAC2-C01a and -C01b DNA vaccines. Each line represents a titration of pre-immune (pre, broken line) or immune (post, solid line) serum from a single rabbit. A) Animals were immunised i.m. into both quadriceps, with two doses of 100  $\mu$ g of DNA vaccine at days 0, 14 and 28. An ear vein bleed was taken at days 0 and 35 and tested in ELISA. B) C01b rabbit was immunised twice more at days 42 and 49. An ear vein bleed was taken at day 56 and tested as before. The responses are shown as specific IgG present in immune compared to pre-immune serum samples.

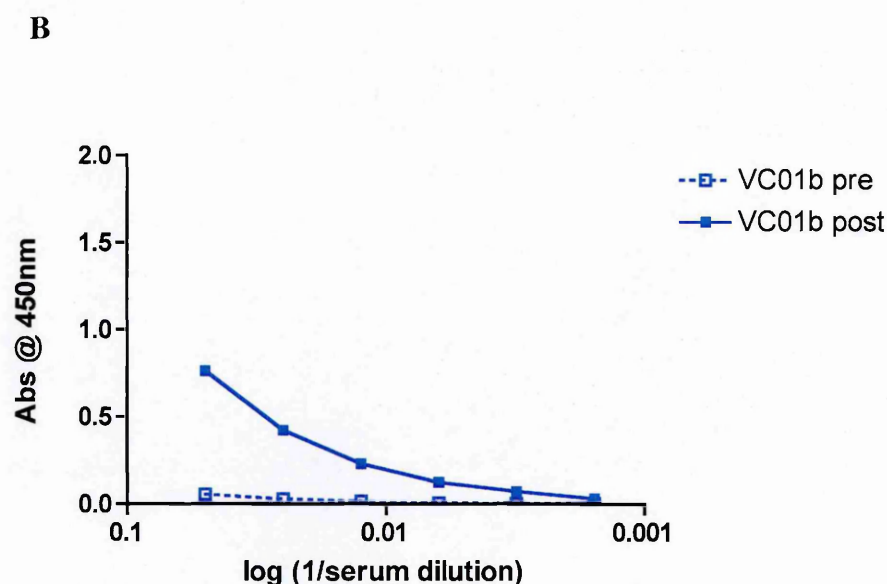
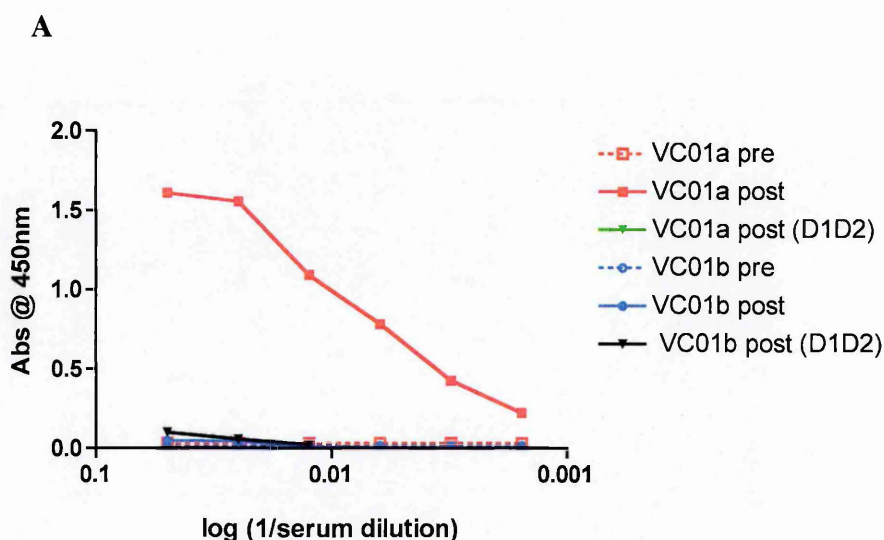


Figure 5.10 Anti-idiotype responses in Dutch rabbits immunised with pVAC2-C01a and -C01b DNA vaccines. Each line represents a titration of pre-immune (pre, broken line) or immune (post, solid line) serum from a single rabbit. A) Animals were immunised i.m. into both quadriceps, with two doses of 100 µg of DNA vaccine at days 0, 14 and 28. An ear vein bleed was taken at days 0 and 35 and tested in ELISA against cognate scFv-CaM and irrelevant D1D2-CaM. B) C01b rabbit was immunised twice more at days 42 and 49. An ear vein bleed was taken at day 56 and tested as before. The responses are shown as specific IgG present in immune compared to pre-immune serum samples.

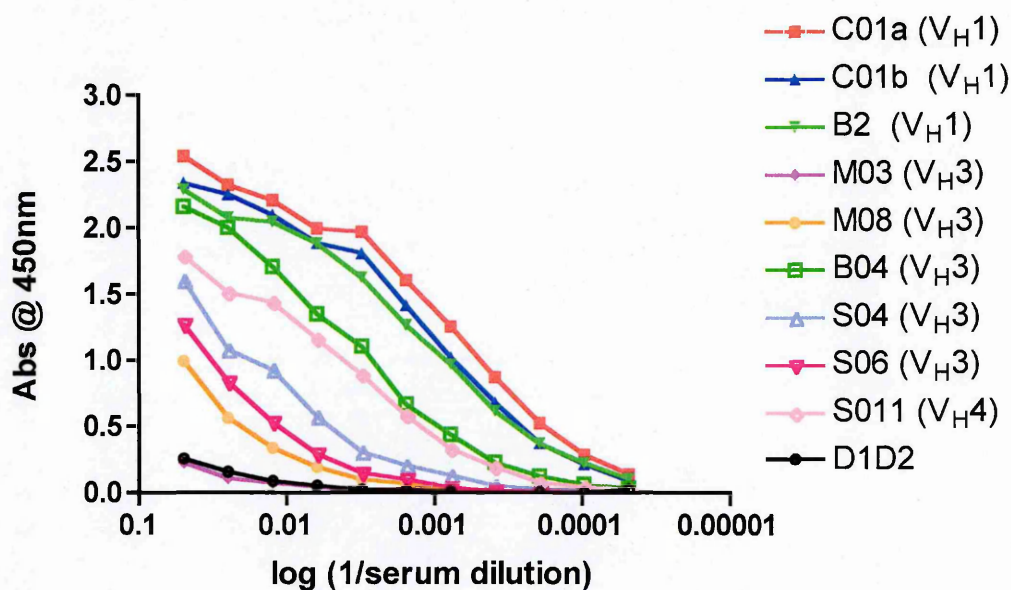


Figure 5.11 Idiotypic cross reactivity of C01a and C01b. Rabbit anti-idiotypic antiserum raised against C01a was tested by ELISA for IgG binding to a panel of 22 CaM tagged scFvs encoded by a range of variable region genes. Binding curves are shown for the seven cross-reactive scFvs. The cognate is shown in red and the negative control irrelevant CaM tagged D1D2 is shown in black.

| Antigen | Germline       |                | % homology     |                |
|---------|----------------|----------------|----------------|----------------|
|         | V <sub>H</sub> | V <sub>L</sub> | V <sub>H</sub> | V <sub>L</sub> |
| C01a    | 1-02           | κ 3-20         | 100            | 100            |
| C01b    | 1-02           | κ 3-20         | 91             | 100            |
| B2      | 1-02           | λ 2-14         | 85             | 46             |
| M03     | 3-23           | λ 2-14         | 47             | 40             |
| M08     | 3-15           | λ 2-08         | 45             | 44             |
| B04     | 3-48           | κ 3-20         | 41             | 88             |
| S04     | 3-21           | κ 3-11         | 46             | 82             |
| S06     | 3-30           | κ 1-09         | 48             | 65             |
| S011    | 4-34           | κ 4-01         | 44             | 70             |
| D1D2    | N/A            | N/A            | ---            | ---            |

Table 5.2 Germline genes encoding each of the scFvs shown in Figure 5.9. Percentage (%) homology at the amino acid level is that between C01V<sub>H</sub>a or C01V<sub>L</sub>a and the V<sub>H</sub>/V<sub>L</sub> genes of each scFv.

### **5.4.7 Relationship between the isolated scFvs and the IgM paraprotein**

To investigate the relationship between C01a/C01b and the patients IgM paraprotein, I investigated whether purified IgM from the patient's serum could inhibit the binding of anti-idiotypic sera to cognate scFv.

#### **5.4.7.1 Purification of patient IgM**

The purified IgM was analyzed on a 12% SDS-PAGE gel (Figure 5.12). 10  $\mu$ l of normal human IgM (1 mg/ml Sigma) was used to assess the quality of the patient IgM. The concentration of protein in the purified fraction was 0.7 mg/ml as determined by BCA assay (Chpt 2.2.2.10).

#### **5.4.7.2 Inhibition of anti-idiotypic serum**

The binding of polyclonal rabbit antibodies against the C01a idiotype to recombinant C01a-CaM was inhibited with purified patient IgM and normal human IgM. In short, C01a-CaM was immobilised on the solid phase and the signal of the rabbit anti-serum was determined in the presence of increasing amounts of purified IgM. The antiserum was used at a final dilution of 1/480, based on the results in Figure 5.11. The purified IgM was used at a range of concentrations (1000 ng/ml to ~2 ng/ml).

The patient's IgM but not control IgM produced 50% inhibition of binding at approximately 5 ng/ml. The control IgM was able to inhibit anti-idiotypic binding to a

similar degree at 100-fold higher concentration suggesting that the relevant idiotype is present in normal human serum, but at very low concentrations (Figure 5.13).

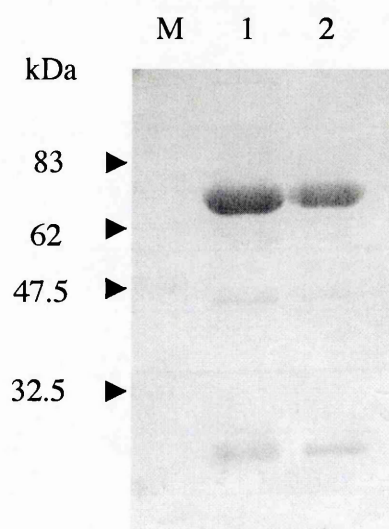


Figure 5.12 Purified IgM from patient serum. IgM was purified from 10 ml of patient serum using an anti-human IgM agarose column. Bound IgM was washed with 20 ml PBS before elution in 1 ml fractions with 0.1 M glycine-HCl pH 2.5. 10  $\mu$ l of the eluted fraction was analyzed on a 12% SDS-PAGE gel. Lane 1 purified patient IgM, lane 2 normal human IgM (Sigma). M broad range molecular weight marker.

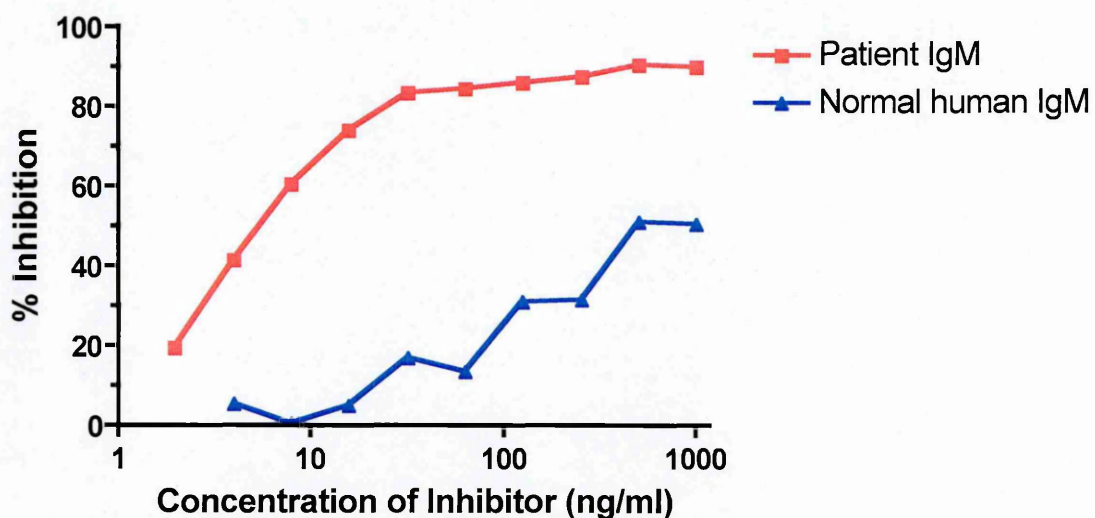


Figure 5.13 Inhibition of the binding of polyclonal anti-C01a (rabbit sera to calmodulin tagged scFv-C01a with IgM purified from the serum of the patient (red) or by normal human IgM (blue) (Sigma). Minimum and maximum concentrations of inhibitor are 2 ng and 1000 ng/ml respectively. The IC50 values were extrapolated from the graph using GraphPad Prism®.



## 5.5 Discussion

The work described in this chapter presents a detailed analysis and characterisation of an IgM  $\kappa$  paraprotein associated with non-Hodgkin's Lymphoma and concomitant severe thrombocytopenia. The patient had a 10-year history of rheumatoid arthritis and had previously been diagnosed with low-grade B cell non-Hodgkin's lymphoma associated with a severe and treatment refractory thrombocytopenia. Her serum was shown to contain an IgM paraprotein with a  $\kappa$  Bence-Jones protein trace in the urine. The initial screen for PAIg and platelet serum antibodies by platelet immunofluorescence at 37°C were both negative, however the severity and therapy refractoriness of the thrombocytopenia and the presence of a paraprotein prompted further investigations at temperatures below 37°C. These studies revealed a potent, IgM  $\kappa$ , platelet antibody (Figure 5.1), with results suggesting that the platelet antibody and the paraprotein were derived from the same (malignant) B cell clone. The results presented define the molecular characteristics of the variable domains of the IgM paraprotein. The idiotypic cross-reactivity between the paraprotein and the platelet IgM was investigated by raising idiotypic specific antibodies in rabbits using tetanus-toxoid enhanced DNA-based idiotypic vaccination (King *et al.*, 1998). Initially, assays were conducted to determine the temperature dependence of the PAIgM with a decrease in binding observed with increasing temperature (Figure 5.2). This pattern of reactivity is very similar to that of cold agglutinins in CHAD patients. Second the serum was reacted with the platelets from a patient with Glanzmann's Thrombasthenia and no binding of IgM was observed, strongly suggestive of  $\alpha$ Ib $\beta$ 3 being the prime target (Figure 5.3). The rearranged V genes were rescued by RT-PCR. When using C $_{\gamma}$  or C $_{\kappa}$  primers bands for all V $_{\text{H}}$  and V $_{\kappa}$  gene families were observed

(Figure 5.4 A and C). However, as expected, using a C<sub>μ</sub> primer V<sub>H</sub> gene amplicons were obtained only in combination with primers of a single V<sub>H</sub> family (V<sub>H</sub>1) (Figure 5.4 D). After depleting the bone marrow aspirate of γ<sup>+</sup> B-cells, prominent amplicons were again obtained with the C<sub>μ</sub> and V<sub>H</sub>1 primers (Figure 5.5 A) and with C<sub>κ</sub> and V<sub>κ</sub>1 and V<sub>κ</sub>3 family primers only (Figure 5.5 B). Sequencing of the cloned amplicons revealed two dominant heavy chain clones both with a sequence highly homologous to the V<sub>H</sub>1-02 (allele 4) germline gene (97.3 and 93.9% at amino acid level) and with identical V<sub>H</sub>-CDR3 sequences (Figure 5.6 A). We assume that all the differences between the germline gene and the bone marrow derived clones were caused by somatic hypermutation or gene rearrangement as sequencing the patients germline gene did not identify novel allelic variants of the V<sub>H</sub>1-02 gene (data not shown). The sequence identity in V<sub>H</sub>-CDR3 and the four common nucleotide mutations between the two V<sub>H</sub> clones is diagnostic of a strong clonal relationship, with both being derived from the same malignant B-cell clone. Sequencing of 10 V<sub>κ</sub>3 clones derived from the RNA of γ<sup>-</sup> B-cells showed a single dominant sequence, which was identical at nucleotide level to the V<sub>κ</sub>3-20-1 germline gene except for two non-synonymous mutations in CDRs 2 and 3 (residues 52 and 93 respectively, Figure 5.6 B). Several studies have shown that the level of somatic hypermutation of rearranged V genes varies between cases of follicular lymphoma. Minimal to no hypermutation is generally associated with a benign outcome and is indicative of the malignant clone being derived from early germinal centre B-cells, whilst a poor outcome is more frequently associated with clones which have undergone extensive hypermutation (Bahler *et al.*, 1991; Zelenetz *et al.*, 1991; Alizadeh *et al.*, 2000). The low level of somatic hypermutation of the rearranged V genes rescued from the malignant clone is compatible with the relatively benign course of the patient's lymphoma.

A number of avenues of investigation were pursued to confirm the hypothesis that the IgM  $\kappa$  paraprotein present in the patient's plasma was the platelet reactive IgM antibody and to determine whether either of the  $V_H$  domains showed preferential platelet binding. First, the rearranged V genes were used to prepare a tetanus toxoid enhanced idiotype vaccine to raise polyclonal idiotypic antibodies against the two dominant clones. Both antisera were specific for the cognate idiotype and were non-reactive with 16/21 irrelevant idiotypes derived from other low grade follicular lymphoma patients or a control antigen. The anti-idiotypic sera were used as probes to investigate the relationship between the paraprotein and the platelet-reactive IgM. Several lines of evidence firmly support that the two populations of antibody are the same. Firstly the platelet reactive antibody was IgM  $\kappa$  (Figure 5.1) and the dominant clones isolated from the bone marrow biopsy were also IgM  $\kappa$  (Figure 5.5). Secondly the binding of the anti-idiotypic serum to its cognate scFv was inhibited by the IgM purified from the patient's serum at low concentration, whilst 100-fold higher levels of purified IgM from a healthy individual were required to achieve the same inhibition (Figure 5.13).

When studying the reactivity of the anti-idiotypic serum against C01V<sub>Ha</sub> with a panel of 21 recombinant scFvs, 5 of the 21 showed reactivity (Figure 5.11). These five positive scFvs are interesting in that they indicate three clusters of reactivity. The anti-C01V<sub>Ha</sub> serum showed an almost equally strong reactivity with the V<sub>H</sub>1-02 encoded scFv-B2 as with the two cognate scFvs, in addition the B2 anti sera reacted equally strongly with both patient derived scFvs. This strong cross-reactivity is best explained by the fact that all three  $V_H$  domains are derived from the V<sub>H</sub>1-02 germline gene and the high level of homology between their sequences (C01V<sub>Ha</sub> versus C01V<sub>Hb</sub>, 91% homology; C01V<sub>Hb</sub> versus B2, 85% homology). It is highly unlikely that the cross-

reactivity is explained by the V<sub>L</sub> domains with B2 having a λ domain and the lymphoma derived IgM a κ. The observation that the strong cross-reactivity is in both direction is interesting against the backdrop of the completely different V<sub>L</sub> domains and the 15% sequence difference between B2 and C01. The latter is mainly due to the somatic hypermutation of the C01 and the B2 V<sub>H</sub> genes and the difference between their respective VH-CDR3.

Germline encoded blood cell antibodies are generally of low affinity and are removed from their target antigen by washing with saline at 37°C (Bye *et al.*, 1992). This was reflected in our inability to demonstrate binding of the monomeric scFv C01 to platelets (data not shown). Identifying the epitope of a low affinity autoantibody is challenging as standard techniques are unsuitable.

Further experiments were performed by Dr Ian Harmer to obtain further evidence that the platelet reactive IgM is indeed encoded by the malignant B cell clone. Firstly F(ab')<sub>2</sub> fragments from anti-idiotypic sera raised against C01a were used in PIFT in place of the anti-human isotype conjugate. Anti-C01a revealed binding of antibodies in the patient's plasma to platelets. Secondly, platelet reactive IgM was purified from the patients plasma. The IgM heavy chain protein band was analysed by Maldi-Tof. The analysis detected two peptides common to both V<sub>H</sub>C01a and V<sub>H</sub>C01b and four unique to V<sub>H</sub>C01b.

In conclusion this study is the first to characterise the V genes encoding a clinically harmful platelet cold agglutinin. The use of minimally mutated V genes in this low affinity platelet IgM mirrors the pattern seen for classical anti-I/i cold agglutinins in CHAD (reviewed in (Pruzanski & Katz, 1984; Roelcke, 1989)). As noted in the clinical summary, the patient's autoimmune thrombocytopenia failed to respond to all conventional and more advanced treatments. Consistent with reports on chronic

haemagglutinin disease, treatment with Rituximab produced a marked clinical response and her platelet counts returned to normal. After six weeks a bone marrow examination was normal with complete remission of her B-cell non-Hodgkin lymphoma on morphological, immunophenotypic, and heavy chain gene rearrangement analyses and the paraprotein band resolved. The response to Rituximab provides additional strong evidence that the paraprotein was causative and the Maldi-Tof analysis indicates that only the V<sub>Hb</sub>/V<sub>κ</sub>3-20 encoded IgM was platelet reactive. The clinical history suggests that the V<sub>Hb</sub> clone emerged at a later stage in the disease coinciding with the acute onset of a profound and treatment refractory thrombocytopenia. Generally the platelet autoantibodies in immune thrombocytopenia in patients with a B-cell lymphoproliferative disorder (Hedge *et al.*, 1985; Kuznetsov *et al.*, 1992; Lim & Ifthikharuddin, 1994) are thought to be produced by autoreactive B-cell clones that have emerged as a consequence of the patients' immunomodulated state (Gupta *et al.*, 2000). All the evidence that we have accrued supports our contention that in this case the IgM paraprotein produced by the lymphoma clone was responsible for the severe thrombocytopenia and such platelet cold agglutinin cases warrant another treatment modality.

The data described in this chapter has been accepted by Transfusion as an original manuscript.

## Chapter 6 – Conclusions

### 6.1 Production of recombinant scFv

The use of the *Drosophila* expression system to produce large quantities of recombinant scFv was investigated in this study. Firstly, I modified the open reading frame of the multiple cloning site of Invitrogen's pMT/BiP/V5 His C so that it was complementary to the DNA vaccine plasmid pVAC2. This modified plasmid is called pMT/BiP/V5 His D (Figure 3.2). Secondly, I inserted the gene for Calmodulin (CaM) in place of the V5/His sequences to produce pMT/BiP/CaM (Figure 3.3). I then compared the expression of four scFv's in both modified plasmids, with pMT/BiP/V5 His D 3/4 lost the hexahistidine tag and were unavailable for purification (Figure 3.12). PMT/BiP/CaM was more successful with all four producing milligram quantities of scFv-CaM (Table 3.1). I also used a bacterial expression plasmid pUC119-His and compared the yields and binding abilities of one scFv, B2 an anti-glycoprotein (GP) IIbIIIa. The yields were far superior using *Drosophila* and with the added benefit of post-translational modifications the decision was taken to continue with the pMT/BiP/CaM construct.

This construct was used to express all twenty-five patient derived scFvs, the results show that one failed and the remaining twenty-four produced yields of between 0.5 mg and 85 mg/L (Table 3.1). I conclude that I have developed a reliable expression system capable of producing large quantities of pure recombinant scFv.

## 6.2 Clinical trial

The clinical trial was ongoing throughout the duration of this project; together with two colleagues we prepared twenty-five clinical grade vaccines to GMP standard (Table 4.5). The vaccines were quality controlled for concentration, purity (260/280 nm ratio), sterility, endotoxin level and sequence integrity (DNA sequencing and restriction enzyme digest). These vaccines were then administered to Dutch rabbits to provide control serum to assess the potential immunogenicity of the vaccines and to establish the ELISA protocols for measuring anti-tetanus and anti-idiotypic responses (Table 4.6).

Once the vaccines had passed the quality controls they were released to the trial centres in Bournemouth, Manchester and Southampton for administration to the patients. For this thesis ten patient serum samples were tested for anti-tetanus (Figure 4.14) and anti-idiotypic responses (Figure 4.13) pre and post vaccination. From this cohort the results suggest that this DNA vaccination regime as a means to produce anti-idiotypic responses in patients with Follicular Lymphoma (FL) is not successful. However, it is known from the research of groups in America that anti-idiotypic antibodies and subsequent patient remissions are attained in patients who have been given recombinant idiotypic vaccinations. Further studies could potentially employ the recombinant scFv-CaM fusions that I produced in *Drosophila* S2 cells as a potential vaccine.

### 6.3 Case Study

The purpose of this study was to provide a unique set of reagents utilizing the recombinant protein expression and DNA vaccination to confirm that the paraprotein derived from the malignant clone of a patient with low-grade FL binds to platelets causing life-threatening thrombocytopenia. I have presented a detailed account of this autoantibody proving that it was derived from the patients' malignant clone. The antigen was also discovered to be the  $\alpha\text{IIb}\beta 3$  integrin. Immunoprecipitation and immunoblot are unsuitable for identifying the epitope of low affinity autoantibodies, we chose a potentially time consuming study utilising platelets from patients lacking expression of individual surface proteins. We obtained platelets from a patient with Glanzmann's Thrombasthenia (GT) and the malignant IgM failed to bind. GT platelets lack expression of the  $\alpha\text{IIb}\beta 3$  integrin; therefore the antigen can be confirmed as the  $\alpha\text{IIb}\beta 3$  integrin.



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## Appendices

### A – scFv nucleotide and amino acid sequences

scFv cloned nucleotide and amino acid sequences between NcoI and Not I restriction sites in pMT/BiP/CaM and pVAC2.

#### B2

```
CCATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAGGCCTGGGGCCGC
      Q V Q L V Q S G A E V K R P G A A
AGTGAAGGTCTCCTGCAAGGCTTCTGGCTACAGATTACCGGCCACTATATGCACTGG
      V K V S C K A S G Y R F T G H Y M H W
GTGCGGCAGGCCCTGGCCAAGGACTTGAGTGGATGGGATGGATCAACCCTAACAGTG
      V R Q A P G Q G L E W M G W I N P N S
GCGGCACGAGCTATGCACAGAAGTTTCAGGGCAGGGTCACCATGACCAGGGACACGTC
      G G T S Y A Q K F Q G R V T M T R D T S
CATCAGCACAGCCTATATGGAGATGACCAGGCTGAGATATGACGACACGGCCGTCTAT
      I S T A Y M E M T R L R Y D D T A V Y
TATTGCGCGGGCGGGAGGTCTAGGGGGCTATTATTACTACGCTATGAACATCTGGGGCC
      Y C A A G G L G G Y Y Y Y A M N I W G
AAGGGACCACGGTCACCGTGTGAGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGG
      Q G T T V T V S S G G G G S G G G G S G
CGGTGGCGGATCGCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGA
      G G G S Q S A L T Q P A S V S G S P G
CAGTCGATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATG
      Q S I T I S C T G T S S D V G G Y N Y
TCTCCTGGTACCAACAGCACCCAGGCAAAGCCCCAACTCATGATTATGAGGTCAG
      V S W Y Q Q H P G K A P K L M I Y E V S
TAATCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCC
      N R P S G V S N R F S G S K S G N T A
TCCCTGACCATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCAGTCTAT
      S L T I S G L Q A E D E A D Y Y C S S
ATACAAGCAGCAGCACTTGGGTGTTGCGGCGGAGGGACCAAGCTGACCGTCCTAGGT
      Y T S S S T W V F G G G T K L T V L G
```

#### B01

```
CCATGGCCAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGAC
      Q V Q L Q E S G P G L V K P S E T
CCTGTCCCTCTCCTGCAGTGTCTCTGGTGCCTCCGTCACTGCTTACTCCTGGAGCTGG
      L S L S C S V S G A S V T A Y S W S W
ATCCGGCAGTCCCCAGACAAGGGACTGGACTGGATTGGTAACATCTCTTACACCGGAA
      I R Q S P D K G L D W I G N I S Y T G
ATACCAAGTACAATCCTTCCGTCAAGAGTCGAGTCATTATTTCACTAGACACGTCCAA
      N T K Y N P S V K S R V I I S L D T S K
GAATCATTTCTCCCTGAAAGTGACGTCTGTGACCGTCGCAGATACGGCCATTTATTAC
      N H F S L K V T S V T V A D T A I Y Y
```

TGTGCGAGGCTCCGGAGCGGCAGCTCGGCCTTTGACTACTGGGGCCAGGGAACCCTGG  
 C A R L R S G S S A F D Y W G Q G T L  
 TCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATC  
 V T V S S G G G G S G G G G S G G G G S  
 GCAGTCTGTGTTGACGCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGCGGGTCACC  
 Q S V L T Q P P S V S G A P G Q R V T  
 GTCTCCTGCACTGGGAGCAGCTCCAACATCGGGGCAGGTTATGATGTACAGTGGTACC  
 V S C T G S S S N I G A G Y D V Q W Y  
 AACAGGTTCCAGGAACAGCCCCAAACTCCTCATCTATGGTAACTTCAATCGGCCCTC  
 Q Q V P G T A P K L L I Y G N F N R P S  
 AGGGGTCCCTGATCGATTCTCTGGCTCCAAGTCTGCCACCTCAGCCTCCCTGGCCATC  
 G V P D R F S G S K S A T S A S L A I  
 ACTGGGCTCCAGGCTGAGGATGAGGCTGAATATTATTGTCAGTCCTATGACGGCAGCC  
 T G L Q A E D E A E Y Y C Q S Y D G S  
 TGAGTGGTGTAAATATTCGGCGGGGGGACCAACCTGACCGTCCTAGGT  
 L S G V I F G G G T N L T V L G

## B02

CCATGGCCGAGATGCAGTTGGTGGAGTCTGGGGGAGGCTTGGTCCAGACGGGGGAGTC  
 E M Q L V E S G G G L V Q T G E S  
 CCTGAGACTCTCCTGTTTCAGCCTCTGGATTCACTATCAGTAGTCACATTATGCATTGG  
 L R L S C S A S G F T I S S H I M H W  
 GTCCGCCAGGCTCCGGGGCAAGGACTGAAATATGTTTCAGGTATTAGTGCTTATGGTG  
 V R Q A P G Q G L K Y V S G I S A Y G  
 ATAAAACATATTACGCAGACTCCATGAGGGACAGATTCGCCATCTCTAGAGACAATTC  
 D K T Y Y A D S M R D R F A I S R D N S  
 CAAGAACACAGTATATCTTCAAATGAGCAGTCTGAGAATTGAGGACACGGCTGTATAT  
 K N T V Y L Q M S S L R I E D T A V Y  
 TACTGTGCGACCAATCACTCGGAAGGCTCTTTTGACTCATGGGGCCAGGGAACCCTGG  
 Y C A T N H S E G S F D S W G Q G T L  
 TCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATC  
 V T V S S G G G G S G G G G S G G G G S  
 GGACATCGTGATGACCCAGTCTCCAGACTCCCTGGTTGTGTCTCTGGGCGAGAGGGCC  
 D I V M T Q S P D S L V V S L G E R A  
 ACCATCAGATGCAAGTCTAGCCACAGTATTTTCATACTATTCCAACAATAAGCACTACT  
 T I R C K S S H S I S Y Y S N N K H Y  
 TAGGTTGGTACCAGCAGAAACCAGGACAGCCTCCTAAGTTACTCATTAATTGGGCATC  
 L G W Y Q Q K P G Q P P K L L I N W A S  
 TACCCGGGAATCCGGGGTCCCTGACCGATTTAGTGGCAGCGGGTCTGGGACAGATTTTC  
 T R E S G V P D R F S G S G S G T D F  
 ACTCTCACCATCAGCAGCCTGCAGACTGAAGATGTGGCAGTCTATTACTGTCAGCAAT  
 T L T I S S L Q T E D V A V Y Y C Q Q  
 ATTATTTTACTCCGTGGACGTTTCGGCCAAGGGACCAAGCTGGAAATCAAACGT  
 Y Y F T P W T F G Q G T K L E I K R



**B03**

CCATGGCCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCT  
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 CCTGAGACTCTCCTGTACAACCTCCGGATTACCTTCAGTAATTATAACATGAGTTGG  
     L R L S C T T S G F T F S N Y N M S W  
 GTCCGCCAGGCTCCGGGGAAGGGGCTGGAATGCATTTACAGACATTAATAGTAGTGGTA  
     V R Q A P G K G L E C I S D I N S S G  
 AAAACATCTTCTACGCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGGGACAATGT  
     K N I F Y A D S V K G R F T I S R D N V  
 CAAGAACGTAGTGTACCTGCAAATGAACAGCCTGAGAGACGAGGACACGGCTGTGTAT  
     K N V V Y L Q M N S L R D E D T A V Y  
 TACTGTGCGAGAGACACTCCGGAGGCACCTGTCTCCGACTACTACGATGTGGACGTCT  
     Y C A R D T P E A P V S D Y Y D V D V  
 GGGGCCAAGGGACCACGGTCACCGTCTGCTCAGGTGGAGGCGGTTACAGGCGGAGGTGG  
     W G Q G T T V T V C S G G G G S G G G G  
 CTCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCA  
     S G G G G S D I Q M T Q S P S S L S A  
 TCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATT  
     S V G D R V T I T C R A S Q G I R N D  
 TAGTCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATA  
     L V W Y Q Q K P G K A P K R L I Y A A Y  
 CAGTTTGCAAGCTGGGGTCCCATCAAGATTCAGCGGCAGTGGATCTGGCACAGAATTC  
     S L Q A G V P S R F S G S G S G T E F  
 ACTCTCACAGTCAGCAGCCTGCAGTCTGAAGATTTTGCAACTTATTACTGTCTCCAAC  
     T L T V S S L Q S E D F A T Y Y C L Q  
 ATCATAATTACCCTCGAACGTTCCGCCAGGGGACCAAGGTGGAAATCAAA  
     H H N Y P R T F G Q G T K V E I K

**B04**

CCATGGAGGTGCAGGTGGTGGAGTCTGGGGGACGGTGGGTACAGCCGGGGGGGTCTCTT  
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 GAGACTCTCCTGTGTAGCCTCTGGATTCAGCTTCAATGCCTATAACATGAAGTGGGTC  
     R L S C V A S G F S F N A Y T M N W V  
 CGCCAGGCTCCAGGGAAGCGACTGGAGTGGATTTCAAACATTACTAGTAGTGGCTTTA  
     R Q A P G K R L E W I S N I T S S G F  
 CTAAAGATTACGCAGACTCTGTGAAGGGCCGATTCACGGTCTCCAGAGACAATGCCAA  
     T K D Y A D S V K G R F T V S R D N A K  
 GAACTCACTGTATCTGCAAATGAACGGCCTGAAAAACGAGGACACGGCTCTTTATTAC  
     N S L Y L Q M N G L K N E D T A L Y Y  
 TGTGTGAGACAAAACCTTCGGAGGATATGTTTTTGATTCTGGGGCCAAGGGACCACGG  
     C V R Q N F G G Y V F D F W G Q G T T  
 TCACCGTCTCTTCAGGTGGAGGCGGTTACAGGCGGAGGTGGCTCTGGCGGTGGCGGATC  
     V T V S S G G G G S G G G S G G G S  
 GGAAATTGTGTTGACGCAGTCTCCAGGAACCCTATCTTTGCCTCCAGGGGAAAGAGCC  
     E I V L T Q S P G T L S L P P G E R A  
 ACTCTCTCCTGCAGGGCCAGTCAGAGTGTTACTAATAATTATTTAGCCTGGTACCAGC  
     T L S C R A S Q S V T N N Y L A W Y Q

AGATACCTGGCCAGGCTCCCAGGCTCCTCATTTCTGGTGCATCCAGCAGGGCCACTGG  
Q I P G Q A P R L L I S G A S S R A T G  
CATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCGCC  
I P D R F S G S G S G T D F T L T I A  
AGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTTACTCACGGA  
R L E P E D F A V Y Y C Q Q Y G Y S R  
CGTTCGGCCAAGGGACCAAGGTGGAAATCAAA  
T F G Q G T K V E I K

## B05

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L R L S C A A S G F T F N N Y D M T W  
GTCCGCCAGGCTCCAGGGAAGGGACTGGAGTGGGTCTCCACTATTAGTGGCAGCGGTG  
V R Q A P G K G L E W V S T I S G S G  
TCAAGACATACTACGCAGACTCTGTGAAGGGTCGCTTCAGCACCTCCAGAGACAACCTC  
V K T Y Y A D S V K G R F S T S R D N S  
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K D T V N V Q M N S L R V E D T A M Y  
TACTGTGCGAAGAATATTAGTATGGAAATATGGGGCCAGGGAACCCTGGTCACCGTCT  
Y C A K N I S M E I W G Q G T L V T V  
CCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCCA  
S S G G G G S G G G G S G G G G S D I Q  
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M T Q S P S S L S A S V G D R V T I T  
TGTCGGGCAGGTCAGGCCATTAGAAATGATTTAGACTGGTATCAGCAGAAACCAGGGA  
C R A G Q A I R N D L D W Y Q Q K P G  
AAGCCCCTAAGCGCCTGATTTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAAG  
K A P K R L I Y A A S S L Q S G V P S R  
GTTTCAGCGGCAGTGGATCTGGGACAGAATTCCTCTCACAATCAGCAGCCTGCAGCCT  
F S G S G S G T E F T L T I S S L Q P  
GAAGATTTTGCAACTTATTACTGTCTACAGCTTAATACTTACCCGCTCACTTTCGGCG  
E D F A T Y Y C L Q L N T Y P L T F G  
GAGGGACCAACGTGAAGATCAAT  
G G T N V K I N

## M01

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L S L I C T V S G G S I S D S N Y F W  
GCCTGGATCCGCCAGCCCCCGGGGAGGGGCTGGACTGGATTGGAAATATTTTCATCTA  
A W I R Q P P G E G L D W I G N I S S  
ATGCGAAGACTTACAATCCGTCCCCCAAGAGTCGGGTACCTTATTCATAGACACGTC  
N A K T Y N P S P K S R V T L F I D T S

CAAAAATCAGTTCTCCCTGAAATTGAGCTCTGTGACCGTCGCAGACACGGCTATTTAT  
 K N Q F S L K L S S V T V A D T A I Y  
 TACTGCGCGAGAGGGAAGTGGACCAACAATTCCTTCGACTTCTGGGGCCAGGGAACCC  
 Y C A R G N W T N N S F D F W G Q G T  
 TGGTCACCGTCTCCTCAGGTGGAGGCGGTTTCATGCGGAGGTGGCTCTGGCGGTGGCGG  
 L V T V S S G G G G S C G G G S G G G G  
 ATCGGACATCCAGATGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGCGAGAGG  
 S D I Q M T Q S P D S L A V S L G E R  
 GCCACCATCAACTGCAAGTCCAGCCAGAGTGTTTTAAATAGGTCCAACAATAAGAACT  
 A T I N C K S S Q S V L N R S N N K N  
 ACTTAGCTTGGTATCAACAAAAGTCAGGACAGCCTCCTAAACTGCTCATTTACTGGAC  
 Y L A W Y Q Q K S G Q P P K L L I Y W T  
 ATCTACCAGGGAATCCGGGGTCCCTGACCGATTTCAGTGGCAGCGGGTCTGGGACAGAT  
 S T R E S G V P D R F S G S G S G T D  
 TTCACTCTCACCATCAGCAGCCTGCAGGCTGAAGATGTGGCAGTATATTACTGTCAGC  
 F T L T I S S L Q A E D V A V Y Y C Q  
 AATCTTTTAGTACTCCGTGCACGTTTCGGCCAAGGGACCAAAGTGGATATCAAACGT  
 Q S F S T P C T F G Q G T K V D I K R

## M02

CCATGGCCCAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGAC  
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 L S L T C T V S G G S I N S S D Y Y W  
 GCCTGGATCCGCCAGCCCCCAGGGAAGGGACTGGAGTGGATTGGAACATCTTTTATA  
 A W I R Q P P G K G L E W I G T I F Y  
 TTGGGAGAACCTACTACAATCCGTCCCTCAAGAGTCGCGTCACCATATCCGGAGACAC  
 I G R T Y Y N P S L K S R V T I S G D T  
 GTCCAAGAACCAATTCTCCCTGCAACTGAGGTCTATGACCGCCGCAGACACGGCTGTG  
 S K N Q F S L Q L R S M T A A D T A V  
 TTTTACTGTGTGAGACAAACTGTCTGGGGATAAGTATCGTTGTGACTTTGACTATTGGG  
 F Y C V R Q T V G D K Y R C D F D Y W  
 GCCAGGGAACCTGGTACCGTCTCCTCAAGTGGAGGCGGTTCAAGCGGAGGTGGCTC  
 G Q G T L V T V S S S G G G S S G G G S  
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 G G G G S D I Q M T Q S P S F L S A S  
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 V G D R V T I T C R A S Q G I S S N L  
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 A W Y Q Q K P G K A P K L L I Y A A S T  
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 L Q S E V P S R F S G S G S G T E F T  
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 L T I N S L Q P E D F A T Y Y C Q Q L  
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 S S Y P V T F G Q G T R L E I K R

**M03**

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L S C V T S G G T F S N H V V T W V R  
CAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAAGTATTAGTGGTAGTGATAGGCGAA  
Q A P G K G L E W V S S I S G S D R R  
CAGATTATGCAGAGTCTGTGAAGGGCCGCTTCAGTTTCTCCAGAGACACTTGCAACAA  
T D Y A E S V K G R F S F S R D T C N K  
AACATTTTTTTCTGCAAATGAACAATCTGACAGTCGCGGACACGGCCGTATATTATTGT  
T F F L Q M N N L T V A D T A V Y Y C  
GCGACTGATCGGACTGAGGGGCACGATTTTTTGAATGGTTCTTATATCAGTGGCGGGC  
A T D R T E G H D F W N G S Y I S G G  
CTCACGGACGAAAGTGTCACAGTTTGGACGTCTGGGGCCAAGGGACCACGGTCACCGT  
P H G R K C H S L D V W G Q G T T V T V  
CTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAATCT  
S S G G G G S G G G G S G G G G S Q S  
GCCCTGACTCAGCCTGCCTCCGTGTCGGGGTCTCTTGGACAGTCGATCACCATTAATT  
A L T Q P A S V S G S L G Q S I T I N  
GCACTGGAACCGGCAGTGACTTTGATGTTTGTGCATCTGTCTCCTGGTACCAACAACA  
C T G T G S D F D V C A S V S W Y Q Q H  
CCCAGGCAAAGTCCCCACACTCATAATATATGATGTCACTAATCGGCCCTCAGGCATT  
P G K V P T L I I Y D V T N R P S G I  
TGTAGTCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATCTCTGGGC  
C S R F S G S K S G N T A S L T I S G  
TCCAGGCTGAGGACGAGGCGGATTATTACTGCGGCTCATATATTAGAACCAACACTCT  
L Q A E D E A D Y Y C G S Y I R T N T L  
GCTGTTTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT  
L F G G G T K L T V L G

**M04**

CCGAGGTGCAGCTGGTGGAGTCTGGGGCAGAACTTGTGAGGTCAGGGACCTCAGTCAA  
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L S C T A S G F N I K D S Y M H W L R  
CAGGGGCCTGAACAGGGCCTGGAGTGGATTGGATGGATTGATCCTGAGAATGGTGATA  
Q G P E Q G L E W I G W I D P E N G D  
CTGAATATGCCCCGAAGTTCAGGGCAAGGCCACTTTTACTACAGACACATCCTCCAA  
T E Y A P K F Q G K A T F T T D T S S N  
CACAGCCTACCTGCAGCTCAGCAGCCTGACATCCGAAGACACTGCCGTCTATTATTGT  
T A Y L Q L S S L T S E D T A V Y Y C  
AATGAGGGGACTCCGACTGGGCCGTACTACTTTGACTACTGGGGCCAAGGCACCCTGG  
N E G T P T G P Y Y F D Y W G Q G T L  
TCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATC  
V T V S S G G G G S G G G G S G G G G S  
GGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTC  
D I Q M T Q S P S S L S A S V G D R V

ACCATCACTTGCCAGGCGAGTCAGGACATTAGCAACTATTTAAATTGGTATCAGCAGA  
 T I T C Q A S Q D I S N Y L N W Y Q Q  
 AACCAGGGAAAGCCCCTAAGCTCCTGATCTACGATGCATCCAATTTGGAAACAGGGGT  
 K P G K A P K L L I Y D A S N L E T G V  
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 P S R F S G S G S G T D F T F T I S S  
 CTGCAGCCTGAAGATATTGCAACATATTACTGTCAACAGTCTGATAATCTCCCATTCA  
 L Q P E D I A T Y Y C Q Q S D N L P F  
 CTTTCGGCCCTGGGACCAAAGTGGATATCAAACGT  
 T F G P G T K V D I K R

## M05

CCGAGGTGCAGCTGGTGGAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAA  
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 GGTCTCCTGCAGGGCCTCTGGAAACACCTTCACCGCCTACTATGTGAACTGGGTGCGA  
 V S C R A S G N T F T A Y Y V N W V R  
 CAGGCCCCCTGGACAAGGGCTTGAGTGGATGGGGCTGATCCACCCTACCAGTGGCGACA  
 Q A P G Q G L E W M G L I H P T S G D  
 CATACTATGAACAGAAATTTTCAGGGCAGGGTCACCATGACCAGGGACATGTCCATCAA  
 T Y Y E Q K F Q G R V T M T R D M S I N  
 CACAGCCTACATGGACCTCTTCTGGCTGACCCCTGACGACACGGCCGTCTATTACTGT  
 T A Y M D L F W L T P D D T A V Y Y C  
 GCGAGTGGTCCCGACACGAAAAAGCCGGCTACTGGGGCCAGGGCACCTGGTCCACCG  
 A S G P D T K K A G Y W G Q G T L V T  
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 V S S G G G G S G G G G S G G G G S Q S  
 TGTGTTGACGCAGCCGCCCTCAGTGTCTGGGGGCCCTGGGCAGAGGGTCACTCTCTCC  
 V L T Q P P S V S G A P G Q R V T L S  
 TGCACTGGGAATAGCACCAACCTCGGGGCAAATTATGATGTGCACTGGTACAAGCAAC  
 C T G N S T N L G A N Y D V H W Y K Q  
 TTCCCGAAACGGCCCCCAAACCTCCTCATTTACGGTAACCGCAAGCGGCCCTCAGGGGT  
 L P E T A P K L L I Y G N R K R P S G V  
 TCCTGATCGATTCTCTGGTTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCACTGGG  
 P D R F S G S K S G T S A S L A I T G  
 CTCCAGGCTGAGGATGAGGCTGCATATTATTGCCAGTCCTATGACTACAGCCTGCGAA  
 L Q A E D E A A Y Y C Q S Y D Y S L R  
 CTGTGGTTTTTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT  
 T V V F G G G T K L T V L G

## M06

CCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAG  
 E V Q L V E S G G G L V Q P G G S L R  
 ACTCTCTTGTGTAGCCTCTGGATTACGTTTCAGTACCTCGGCCATGAACTGGGTCCGC  
 L S C V A S G F T F S T S A M N W V R  
 CAGGCTCCAGGGAAGGGCCTGGAGTGGGTCTCACTTATGACTAGTGGTACTATTACAC  
 Q A P G K G L E W V S L M T S G T I T

ATTACGCAGACTCCGTGAAGGACCGATTACCATCTCCAGAGACAATTCCAAGACTAC  
 H Y A D S V K D R F T I S R D N S K T T  
 GTTGTTTTTGCAAATGAACAGCCTGAGAGTCGAGGACACGGCCACATACTACTGTGCG  
 L F L Q M N S L R V E D T A T Y Y C A  
 AATGGTTTCGGACTGGTTCGACCCCTGGGGCCAGGGCACCCTGGTCACCGTCTCCTCAG  
 N G S D W F D P W G Q G T L V T V S S  
 GTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCCAGATGAC  
 G G G G S G G G G S G G G G S D I Q M T  
 CCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGCGAGAGGGCCACCATCAACTGCAAG  
 Q S P D S L A V S L G E R A T I N C K  
 TCCAGCCAGAGTATTTTTTTCCTGGTCCTACAATAAGAACAACCTAACTTGGTATCAGC  
 S S Q S I F S W S Y N K N N L T W Y Q  
 AGAAACCAGGACAGCCTCCTAAATTGCTCATTTACTGGGCATCTACCCGGGAATCCGG  
 Q K P G Q P P K L L I Y W A S T R E S G  
 GGTCCCTGACCGATTCAAGTGGCAGCGGGTCTGGGACAGATTTCACTCTCACCATCAGC  
 V P D R F S G S G S G T D F T L T I S  
 AGCCTGCAGACTGAAGATGTGGCAGTTTATTATTGTCAACAATATTATAGTCGTCCGC  
 S L Q T E D V A V Y Y C Q Q Y Y S R P  
 TCACTTTTCGGCGGAGGGACCAAAGTGGATATCAAACGT  
 L T F G G G T K V D I K R

## M08

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTAAGGCCTGGGGGGTCCCTTAGAC  
 E V Q L V E S G G G L V R P G G S L R  
 TCTCTTGTGTAGGCTCTGGATTCACTTTTAGTAATGCCTGGATGACCTGGGTCCGCCA  
 L S C V G S G F T F S N A W M T W V R Q  
 GGGTCCAGGAAAGGGGCTAGAATGGGTGGTTCGTATTAAAAACATAAGTGAAGGTGGG  
 G P G K G L E W V G R I K N I S E G G  
 ACAACAGACTACGCTACATCCGTTAAAGGCAGATTTTACATCTCAAGAGATGATTCAA  
 T T D Y A T S V K G R F Y I S R D D S  
 AAAATATGGTGTATCTGCAAATGGACAGCCTGAAAACCGAGGACACAGCCATTTATTA  
 K N M V Y L Q M D S L K T E D T A I Y Y  
 TTGTTCCACAAATGTGACCGGACCTCGAGCGCCAATTATGCACTTTGAGTCTTGGGGC  
 C S T N V T G P R A P I M H F E S W G  
 CAAGGCACCCTGGTCACCGTCTCCTCGGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTG  
 Q G T L V T V S S G G G G S G G G G S  
 GCGGTGGCGGATCGCAGTCTGTGCTGACTCAGCCACCCTCCGCGTCCGGGTCTCCTGG  
 G G G G S Q S V L T Q P P S A S G S P G  
 ACAGTCAGTCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTAT  
 Q S V T I S C T G T S S D V G G Y N Y  
 GTCTCCTGGTACCAACAACACCCAGGCAAAGCCCCCAAACCTCATGATTTTTGAGGTCA  
 V S W Y Q Q H P G K A P K L M I F E V  
 ATAAGAGGCCTTCAGGGTCCCTGATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGC  
 N K R P S G V P D R F S G S K S G N T A  
 CTCCCTGACCGTCTCTGGGCTCCAGGCTGAGGATGAAGCTGATTATTATTGCAGCTCA  
 S L T V S G L Q A E D E A D Y Y C S S

TATGCAGGCACCAACACAGTATTTGGCGGAGGGACCAAGCTGACCGTCCTAGGT  
Y A G T N T V F G G G T K L T V L G

## S01

CCCAGGTGCAGTTACAGCAGTGGGGCGCGGGACTCTTGAAGCCGTCGGAGACCCTGTC  
Q V Q L Q Q W G A G L L K P S E T L S  
TCTCATCTGCGCTGTCTATGGGGGATCCCTCAATGATTACTACTGGAGTTGGATCCGC  
L I C A V Y G G S L N D Y Y W S W I R  
CAGCCCCCGGGGGGGGGCCTGGAGTGGCTTGGAGAAGTCGATTCTCGTGGAGGCACCA  
Q P P G G G L E W L G E V D S R G G T  
ACTACAGCCTGGCCCTCAGGGGTCGCGTCACGGTGTCTTAGACATGTCCAAGAACTC  
N Y S L A L R G R V T V S L D M S K N S  
CTTCTCCCTAAAATTGACCTCTGTGACCGTCGCGGACACGGCTACCTATTTCTGCGCG  
F S L K L T S V T V A D T A T Y F C A  
AGAGGACTTGGTCTAGACATGGA CTTCGATCTCTGGGGCCGTGGCACCCTGGTCACCG  
R G L G L D M D F D L W G R G T L V T  
TCTCCTCAGGTGGAGGCGGTT CAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAAAAG  
V S S G G G G S G G G G S G G G G S K S  
TGTGCTGACGCAGTCTCCAGACACCCTGTCTTTGTCTCCAGGGGAAAACGCCACCCTC  
V L T Q S P D T L S L S P G E N A T L  
TCCTGTAGGGCCAGTCAGACGGTTGACGGCAACTTCTTAGCCTGGTATCAGCAAAAAC  
S C R A S Q T V D G N F L A W Y Q Q K  
CTGGCCAGGCTCCCAGGCTCCTCCTTTACGATGCTTCCACCAGGGCCACTGGCATCCC  
P G Q A P R L L L Y D A S T R A T G I P  
AGACAGGTT CAGTGGCAGTGGGTCTGGGAGAGACTTCACCCTCACCATCAGCAGTCTG  
D R F S G S G S G R D F T L T I S S L  
GAGCCTGAGGATCTCGGAATATACTATTGT CAGA ACTATGATAAGTCTTTGATGTACA  
E P E D L G I Y Y C Q N Y D K S L M Y  
CCTTTGGCCAGGGGACCAAGCTGGAGATCAAA  
T F G Q G T K L E I K

## S02

CCGAGGTACAATTAGTGGAGTCTGGGGGAGGCCTGGTCAAGCCGGGGGGGTCCCTGAG  
E V Q L V E S G G G L V K P G G S L R  
ACTCTTCTGTGCGCCTCTGGATTACCTTCAGAAGTTATACCATGAACTGGGTCCGC  
L F C A A S G F T F R S Y T M N W V R  
CAGGCTCCGGGAAGGGTCTGGAGTGGGTCTCATCTATTAGTGGTTTTAGTTCTTACA  
Q A P G K G L E W V S S I S G F S S Y  
TATACTATGCAGACTCACTGAGGGGCCGATTACCATCTCCAGAGACAACGCCAGAAA  
I Y Y A D S L R G R F T I S R D N A R N  
TTCACTGTCTCTGAAATCAACGACCTGCGACCCGACGACACGGCTATATATTATTGT  
S L S L Q I N D L R P D D T A I Y Y C  
GCGACAAATAGCACCTCGTCGAAGACATCGTACACCTTTGACTACTGGGGCCGGGGAA  
A T N S T S S K T S Y T F D Y W G R G  
CCCTGGTCACCGTCTCCTCAGGTGGAGGCGGTT CAGGCGGAGGTGGTTCTGGCGGTGG  
T L V T V S S G G G G S G G G G S G G G  
CGGATCGCAGTCTGTGCTGGCTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGG  
G S Q S V L A Q P P S A S G T P G Q R

GTCATCATCCCTTGTCTCTGGAAGCAGCTCCAACATCGGAACTAATACTGTAGACTGGT  
V I I P C S G S S S N I G T N T V D W  
ATCAACAATCCCAGGAACGGCCCCCAAACCTCCTCATCTACAGTAATAATCAGCGGCC  
Y Q Q F P G T A P K L L I Y S N N Q R P  
CTCGGGGGTCCCTGACCGATTCTCTGGCTCCAACCTCTGGCACCTCAGCCTCCCTGGCC  
S G V P D R F S G S N S G T S A S L A  
ATCAGTGGGCTCCAGTATGAGGATGAGGCTGACTATTATTGTGCAACACGAGATGTCA  
I S G L Q Y E D E A D Y Y C A T R D V  
ACCTGGATGCTCCGGTGTTCGGCGGAGGGACTAAACTGACCGTCCTA  
N L D A P V F G G G T K L T V L

### S03

CCCTGTTATATCTGGAGGAGTCTGGGGGAGGCTTGGTCAACCCTGGAGGGTCCCTGAG  
L L Y L E E S G G G L V N P G G S L R  
ACTCTCCTGTGCAGCCTCTGGCCTCACCTTCAGGGACTACAATATGAGTTGGATCCGC  
L S C A A S G L T F R D Y N M S W I R  
CAGGCTCCAGGGAAGGGGCTGGAATGGGTTTCAAATATTACTACTAGTGGTAACACCA  
Q A P G K G L E W V S N I T T S G N T  
TAGACTACGCAGACTCTGTGAAGGGCCGATTACCATCTCCAGGGACAACGCCAAGAA  
I D Y A D S V K G R F T I S R D N A K N  
CTCACTGCATCTGCAGATGAACAACCTTGAGAGGCGACGACACGGCCGTATATTATTGT  
S L H L Q M N N L R G D D T A V Y Y C  
GCGACAACAGTGCCTTCTGCTTCTTCTCCTGCCTATTACTACATGGACGTCTGGGGCA  
A T T V P S A S S P A Y Y Y M D V W G  
AAGGGACTACGGTACACGGCTCCTCATGTGGAGGCGGTTCAAGCGGAGGTGGTTCTGG  
K G T T V T G S S G G G G S G G G G S G  
CGGTGGCGGATCGAAAATTATATTGACACAGTCTCCAGCCAACCTGTCTTTGTCTCCA  
G G G S K I I L T Q S P A N L S L S P  
GGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTCTTAGTAGTTCCTTAGCCT  
G E R A A T L S C R A S Q S L S S S L A  
GGTACCAACAAAAACCTGGCCAGGCTCCCAGGCTCCTCATCCATGATGCATCCAACAG  
W Y Q Q K P G Q A P R L L I H D A S N R  
GGCCACTGGCATCCCAGCCAGGTTTCAGTGGCAGTGGGTCTGAGACAGACTTCACTCTC  
A T G I P A R F S G S G S E T D F T L  
ACCATCAGCAGTCTAGAGCCTGAAGATATTGCAGTTTATTACTGTGACGAGCGGGTCA  
T I S S L E P E D I A V Y Y C Q Q R V  
CTTTCGGCGGAGGGACCAAGGTAGAGATCAAA  
T F G G G T K V E I K

### S04

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGAC  
E V Q L V E S G G G L V Q P G G S L R  
TCTCCTGTGCAGCCTCTGGATTACCTTCAGTAGTTATAGCATGAACGGGTCCGCCA  
L S C A A S G F T F S S Y S M N W V R Q  
GGCTCCAGGGAAGGGGCTGGAGTGGATTTCAAACATTAGTAGTAGTATTAGTAACATA  
A P G K G L E W I S N I S S S I S N I



TACTACGCAGACTCTGTGAAGGGCCGATTACCATTTCCAGAGACAATGCCAAGAACT  
Y Y A D S V K G R F T I S R D N A K N  
CACTGTATCTGCAAATGAACAGCCTGAGAGACGAGGACACGGCTGTGTATTACTGTGC  
S L Y L Q M N S L R D E D T A V Y Y C A  
GAGAGATTCCTTGGGCAGTAGCCTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC  
R D S L G S S L D Y W G Q G T L V T V  
TCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCGGAAATTG  
S S G G G G S G G G G S G G G G S E I  
TGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAAGGGAAAATGTCACCCTCTC  
V L T Q S P A T L S L S P R E N V T L S  
CTGCAGGGCCAGTCAGAGTGTTACCAACTACTTAGCCTGGTACCAACAGAAGCCTGGC  
C R A S Q S V T N Y L A W Y Q Q K P G  
CAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCA  
Q A P R L L I Y D A S N R A T G I P A  
GGTTCAGTGGCAGTGGGTATGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCC  
R F S G S G Y G T D F T L T I S S L E P  
TGAAGATTTTGCAGTTTATTACTGTCAACAACGTAGCAACTGGCTGTCTTTCGGCGGA  
E D F A V Y Y C Q Q R S N W L S F G G  
GGGACCAAAGTGGAGATCAAA  
G T K V E I K

**S05**

GAGGTGCAGTTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGAC  
E V Q L V E S G G G L V Q P G G S L R  
TCTCCTGTGCAGCCTCTGGATTACCTTCAGTTCTTATAGCATGACCTGGGTCCGCCA  
L S C A A S G F T F S S Y S M T W V R Q  
GGCTCCAGGGAAGGGGCTGGAGTGGGTTTCAAACATTAGCAGTAGTAGTAATACCGCA  
A P G K G L E W V S N I S S S S N T A  
TTCTACGCAGACTCCGTGAAGGGCCGATTACCATTTCCAGAGACAATGTCAAGAATT  
F Y A D S V K G R F T I S R D N V K N  
CACTGTATCTGCAAATGCACAGCCTGAGAGACGAGGACACGGCTGTATACTTTTGTGC  
S L Y L Q M H S L R D E D T A V Y F C A  
GAGAGATCGAAATGGCTACAATTTCTTTGACTACTGGGGCCAGGGAACCCTGGTCACC  
R D R N G Y N F F D Y W G Q G T L V T  
GTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCGGACA  
V S S G G G S G G G S G G G S D  
TCGAAATGACCCAGTCTCCATCGTCCCTGTCTGCATCTATTGGGGACAGACTCACCAT  
I E M T Q S P S S L S A S I G D R L T I  
CACCTGCCGGGCAAGTCAGGCTATTTCGTGAGTCTGTACAGTGGTATCAACAGAAACCA  
T C R A S Q A I R E S V Q W Y Q Q K P  
GGGAAAGCCCCTAAACTCCTTATGTTTTCAACATCTATTTTGGAAAGTGGTGTCCCGT  
G K A P K L L M F S T S I L E S G V P  
CAAGGTTTCAGCGGCAGTGGATTTGGGACACATTTTACTCTCACCATCAGCAGTCTACA  
S R F S G S G F G T H F T L T I S S L Q  
ACCTGAAGATTTTGCAACTTATTCGTGTCAGCAGAATTACAGTGGCCCTTTCACTTTC  
P E D F A T Y S C Q Q N Y S G P F T F  
GGCCCCGGGACCAAGGTTGAAATGAGA  
G P G T K V E M R

## S06

CAAGTTCAACTAGTGGAGTCTGGGGGCGGCGTGGTCCAGCCTGGGACGTCCCTGAGAC  
 Q V Q L V E S G G G V V Q P G T S L R  
 TCTCCTGTGCAGCCTCTGGATTCCCCTTCATCTACTATGGCATACTGGGTCCGCCA  
 L S C A A S G F P F I Y Y G I H W V R Q  
 GGCTCCAGGCAAGGGGCTGGAGTGGGTTGCAGGTCTCTCGTGTGATGGAACATAATGAA  
 A P G K G L E W V A G L S C D G T N E  
 TATTATGCTGACTCCCTGAAGGGTCGCTTCGTTCATCTCCAGAGACAACCTCCAAGAACA  
 Y Y A D S L K G R F V I S R D N S K N  
 CGGTGTCTCTGCATATGAACCTCTTGAAAACCTGAGGACACGGCTGTATATTATTGTGC  
 T V S L H M N L L K T E D T A V Y Y C A  
 GAAAGATTTTACCAACATTATGGGTCAGGGAGTTATTATCAGGGCACCTTTGACTTT  
 K D F H Q H Y G S G S Y Y Q G T F D F  
 TGGGGCCAGGGAAGCCTGGTCCCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTG  
 W G Q G S L V T V S S G G G G S G G G  
 GTTCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGC  
 G S G G G G S D I Q M T Q S P S S L S A  
 ATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAACAAT  
 S V G D R V T I T C R A S Q S I S N N  
 TAAACTGGTATCAGCAAAAACCAGGGAAACCCCTAGGCTCCTAATCTATGTTGCAT  
 L N W Y Q Q K P G K P P R L L I Y V A  
 CCAGTTTGCAAAGTGGGGTCCCATCAAGGTTCAAGTGGCAGTGGATCTGGGACAGATTT  
 S S L Q S G V P S R F S G S G S G T D F  
 CACTCTCACCATCAGGAGTCTGCAACCTGAAGATTTTGCAACTTATTACTGTCAACAA  
 T L T I R S L Q P E D F A T Y Y C Q Q  
 AATTACAGTCTTTTGCCGCACACTTTTGGCCAGGGGACCAAGCTGGAGATCAGA  
 N Y S L L P H T F G Q G T K L E I R

## S07

GAGGTGCAGTTGGTGGAGTCGGGGGGAGGCTTGGTTTCAGCCGGGGGGGTCCCTGAGAC  
 E V Q L V E S G G G L V Q P G G S L R  
 TCTCCTGTGAAGCCTCTGGCTTCAGCTTCAGTACTTACAGCATGAACGGGTCCGCCA  
 L S C E A S G F S F S T Y S M N W V R Q  
 GGTTTCAGGGAAGGGACTAGAGTGGCTTTCAAACATTAGTAGTACAAGTGGGAAGCACA  
 V S G K G L E W L S N I S S T S G S T  
 AATTACGCAGACTCTGTGACGGCCCGGTTTACCATTTCAGAGACAATGCCCAGAATA  
 N Y A D S V T A R F T I S R D N A Q N  
 CACTGTGGCTACAGATGAACAGCCTGAGAGCCGAGGACACGGCTATATATTACTGTGC  
 T L W L Q M N S L R A E D T A I Y Y C A  
 GAGGACATCTTGTAGTGGTTCGTTCTGTCACTTTGACTACTGGGGCCAGGGAACCCTG  
 R T S C S G R S C H F D Y W G Q G T L  
 GTCACCGTCTTCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGAT  
 V T V F S G G G G S G G G G S G G G G  
 CGGACATCCTGCTGACCCAGTCTCCATCCTCCCTGTCTGCGTCTGTTGGAGACAGAGT  
 S D I L L T Q S P S S L S A S V G D R V  
 CACCATCACTTGCCGGACAAGTCAGAGTATTTACACCTCTTTAAATTGGTTTCAGCAG

T I T C R T S Q S I Y T S L N W F Q Q  
 AAACCGGGGAAAGCCCCTAAACTCCTGATCTATTCTGCATCCACTTTGCAAAGTCAAG  
 K P G K A P K L L I Y S A S T L Q S Q  
 TCCCATCAAGGTTCAAGTGGCAGTGGGTCTGGGACAGATTTCACTCTCACCATCAGCGG  
 V P S R F S G S G S G T D F T L T I S G  
 TCTGCAACCTGAAGATTTTGCAACTTACTATTGTCAACAAAGTTACAGTGCCCTGTAC  
 L Q P E D F A T Y Y C Q Q S Y S A L Y  
 ACTTTTGGCCAGGGGACCAAACTGGAGATCAA  
 T F G Q G T K L E I K

## S09

GAGCTGCAACTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGAC  
 E L Q L V E S G G G L V Q P G G S L R  
 TCTCCTGTGTAGCCTCTGGATTCAATTTTACGAATTATAATATGAGTTGGGTCCGCCA  
 L S C V A S G F N F T N Y N M S W V R Q  
 GGCTCCAGGAAAGGGCCTGGAGTGGATTTCTGTACATAAGCAACACTAGTACGAGCATA  
 A P G K G L E W I S Y I S N T S T S I  
 TACTACGCAGATTCTGTGAAGGGCCGATTCATCATCTCCAGAGACAATGCCAAGGAAT  
 Y Y A D S V K G R F I I S R D N A K E  
 CTCTGTATCTGCAAATGAGCAGCCTGAGAGACGAGGACACGGCTGTCTATTACTGTGC  
 S L Y L Q M S S L R D E D T A V Y Y C A  
 GAGACGGAATGTGACAACGGTGAATCTCGGACTTGATGTTTTTGATATCTGGGGCCAA  
 R R N V T T V N L G L D V F D I W G Q  
 GGGACAATGGTCAACGCTCTCTTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCG  
 G T M V T V S S G G G G S G G G G S G  
 GTGGCGGATCGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCTTCTGTTGG  
 G G G S D I Q M T Q S P S S L S A S V G  
 CGACAGAGTCACCGTCACTTGCCGGGCAAGTCAGAATATAAGTAATTACTTAAATTGG  
 D R V T V T C R A S Q N I S N Y L N W  
 TATCAACTGAAGCCAGGGAAAGTCCCTAAACTCCTGATCTTTGCTGCATCCAGTTTGC  
 Y Q L K P G K V P K L L I F A A S S L  
 AAAGTGGGGTCCCATCAAGGTTCAAGTGGCMGTCAATCTGGGGCAGATTTYACTCTCAC  
 Q S G V P S R F S G S Q S G A D F T L T  
 CATCAACAATCTGCAACCTGAAGATTTTGCAAGTTATTACTGTCAACAGAGTTACAGT  
 I N N L Q P E D F A S Y Y C Q Q S Y S  
 TCCCCTTCGTGGACGTTTCGGCCAAGGGACTAACGTGGAGATCAAC  
 S P S W T F G Q G T N V E I K

## S011

CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGACTTCGGAGACCCTCTCCC  
 Q V Q L Q Q W G A G L L K T S E T L S  
 TCACCTGCGCTGTCTATGGTGGGTCCCTTCAATAATTACAACCTGGACATGGATCCGCCA  
 L T C A V Y G G S F N N Y N W T W I R Q  
 GCCCCAGGGAAGGGCCTGGAGTGGATTGGACAAATTAATCATAGTGAACCACCAAT  
 P P G K G L E W I G Q I N H S G T T N  
 TATAATCCGTCCCTCAAGAGTCGAGTCACCATGTCAATAGACCCGTCCGAGAACCAGT  
 Y N P S L K S R V T M S I D P S E N Q

TCTCCCTGAAGGTGAGATCTGTGACCGCCGCGGACACGGCTATTTATTACTGTGTGAG  
F S L K V R S V T A A D T A I Y Y C V R  
AGGCTCCCCGAATCTTCTGGCAATTACTGGGGACACTTCCAGTATTGGGGCCAGGGC  
G S P E S S G N Y W G H F Q Y W G Q G  
ACCCTGGCCACCGTCTCTTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTG  
T L A T V S S G G G G S G G G G S G G  
GCGGATCGGACATCGTGATGACCCAGTCTCCAGACTCCCTGTCTGTGTCTCTGGGCGA  
G G S D I V M T Q S P D S L S V S L G E  
GAGGGCCACCATCAACTGCAAGTCCAGCCAGAGTGTTTTATACAGCTCCCACAATAAG  
R A T I N C K S S Q S V L Y S S H N K  
AACTACTTAGCTTGGTACCAACAGAAACCAGGACAACCTCCTAGGTTGCTCATTTATT  
N Y L A W Y Q Q K P G Q P P R L L I Y  
GGGCATCTACCCGGAATCCGGGGTCCCTGACCGATTTCAGTGGCAGCGGGTCTGGGAC  
W A S T R E S G V P D R F S G S G S G T  
AGATTTCACTCTCACCATCAACACCCTGCAGGCTGAAGATGTGGCAGTTTATTACTGT  
D F T L T I N T L Q A E D V A V Y Y C  
CAGCAATATTATACTACTCCGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA  
Q Q Y Y T T P Y T F G Q G T K L E I K

## S012

CAGATCACCTTGAGGGAGTCTGGTCCTACGCTGGTGAAACCCACACAGACCCCTCACAC  
Q I T L R E S G P T L V K P T Q T L T  
TGACCTGTACCTTCTCTAATTTCTCACTCACTACTCCTGGAATGGGTGTGGGCTGGGT  
L T C T F S N F S L T T P G M G V G W V  
CCGTCAGCCCCCAGGAAAGGCCCTAGAGTGGCTTGCAGTCGTTTATTGGAATGATTAT  
R Q P P G K A L E W L A V V Y W N D Y  
AAGCGCTACAGCCCATCTCTGAAGACTAGACTCTCCATCACCGCGGACATTTCCAAAA  
K R Y S P S L K T R L S I T A D I S K  
ATCAGGTGGTCCTTACAATGACCAACATGGACCCTGTGGACACAGCCACATATTACTG  
N Q V V L T M T N M D P V D T A T Y Y C  
TACATACAACTCATCGGATCGCATCAAATTCGACCCCTGGGGCCAGGGAATTCGGGTCT  
T Y N S S D R I K F D P W G Q G I R V  
ACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGG  
T V S S G G G G S G G G G S G G G G S  
ATTTTGTGTTGACACAGTCTCCAGGCACCCTGTCTGCATCTGTTGGAGACAGAGTCAA  
D F V L T Q S P G T L S A S V G D R V N  
TATCACTTGCCGGGCCAGTCAGAGTGTTAGTAGGTGGTTGGCCTGGTATCAGCAGAAG  
I T C R A S Q S V S R W L A W Y Q Q K  
CCAGGGAAAGTCCCTGAACTTCTGATCTATGAGGCATCTAATTTAGAGAATGGGGTCC  
P G K V P E L L I Y E A S N L E N G V  
CATCAAGGTTTCAGCGGCAGTGGATCTGGGACAGAATTCAATCTCACCATCAACAGCCT  
P S R F S G S G S G T E F N L T I N S L  
GCAGCCTGATGATTTTGCAACTTATTACTGCCAACAGTATAATAGTTATTGGACATTC  
Q P D D F A T Y Y C Q Q Y N S Y W T F  
GGCCAAGGGACCCGGGTGGAAATCAAA  
G Q G T R V E I K

**S013**

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGTCTGGAGGGTCCCTGAGAC  
 E V Q L V E S G G G L V Q S G G S L R  
 TCTCCTGTGCAGCCTCTGGATTACCTTCAGTTATTATGGAATGAACTGGGTCCGCCA  
 L S C A A S G F T F S Y Y G M N W V R Q  
 GGCTCCAGGAAAGGGGCTGGAGTGGGTCTCCTACATTAGTGGTAGTGGAGGAGCCATT  
 A P G K G L E W V S Y I S G S G G A I  
 TACTACGCAGACTCTCTGAAGGGCCGATTTCATCATCTCCAGAGACAACGCCAAGAACT  
 Y Y A D S L K G R F I I S R D N A K N  
 CACTATATCTGCAAATGAACAACCTGAGAGCCGAAGACACGGCTATTTATTACTGTGC  
 S L Y L Q M N N L R A E D T A I Y Y C A  
 GAGTAATATATCAACTCGGACCTCGTACTTCTTTGACTCCTGGGGCCAGGGAACCCCTG  
 S N I S T R T S Y F F D S W G Q G T L  
 GTCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGAT  
 V T V S S G G G G S G G G G S G G G G  
 CGGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTGTGTCTCCAGGCCAAAGAGT  
 S E I V L T Q S P G T L S V S P G Q R V  
 CACCCTCTCTTGCAGGGCCAGTCAGAGCGTTATCAGCCCATACTTAGCCTGGTTCCAG  
 T L S C R A S Q S V I S P Y L A W F Q  
 CAAAACCTGGCCAGGCTCCAGTCTCGTCATCTATGGTACATCCAGCAGGGCCACTG  
 Q K P G Q A P S L V I Y G T S S R A T  
 GCATCTCAGACAGGATCAGTGGCAGTGGGTCTGGGACAGACTTCAGTCTCACCATCAG  
 G I S D R I S G S G S G T D F S L T I S  
 CAGACTGCAGCCTGAAGATTTTGGAAATATATTACTGTCAGCATTATGGAAGTTCACCT  
 R L Q P E D F G I Y Y C Q H Y G S S P  
 CCGATGTGGACGTTTCGGCCAAGGGACCAAGGTGGAAATCAAC  
 P M W T F G Q G T K V E I N

**S017**

GAGAAACAAGTTTTGGAGTCTGGGGGAAAGTTGGTCCAGCCGGGGGGGTCCCTGAGAC  
 E K Q V L E S G G K L V Q P G G S L R  
 TCTCCTGTGTTGCCTATGGATTCACTTTTGAGAAAAATGCCATCAACTGGGTCCGCCA  
 L S C V A Y G F T F E K N A I N W V R Q  
 GGCTCCAGGGAAGGGGCTGCAGTGGGTCTCAGGTCTTAGTGGTGGTGATTTCATCCACA  
 A P G K G L Q W V S G L S G G D S S T  
 TATTATGCAGACTCCGTGAAGGGCCGTTTCACCATCTCCAGAGACAATTCCGACAACA  
 Y Y A D S V K G R F T I S R D N S D N  
 TGTTTTATTTGCAAATGAGCAGCCTGAGAGTCGAGGACACGGCCTTATATTACTGTGC  
 M F Y L Q M S S L R V E D T A L Y Y C A  
 GACGACTTGCCCTAGTTCCAAGTACTCTTTGTTCATATGCTTTTGATATCTGGGGCCGA  
 T T C P S S K Y S L S Y A F D I W G R

GGGACAATGGTCACCGTGTCTTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCG  
 G T M V T V S S G G G G S G G G G S G  
 GTGGCGGATCGCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACA  
 G G G S Q S A L T Q P A S V S G S P G Q  
 GTCGATCACCATCTCCTGTACTGGGTCCCGCAGTGACATTGGTGGTTACAACCTATGTC

S I T I S C T G S R S D I G G Y N Y V  
 TCCTGGTACCAACAACACCCAGGCAAAGTCCCCAAAGTCATCATTTTTGAAGTCACTT  
 S W Y Q Q H P G K V P K V I I F E V T  
 GTCGGCCCTCAGGGATTTCTTCTCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTC  
 C R P S G I S S R F S G S K S G N T A S  
 CCTTACTATCTCTGGCCTCCAGGCTGACGACGAGGCTCATTATTACTGCAGCTCATAT  
 L T I S G L Q A D D E A H Y Y C S S Y  
 TCAAACAGTCGCACTCGTGTCTTATTCGGCGGAGGGACCAGGTTGACCGTCCTA  
 S N S R T R V L F G G G T R L T V L

## S018

GGGGTGCAGTTGGTGAATCTGGGGGAGGGTTGGTAAAGCCTGGGGGGTCCCTCAGAC  
 G V Q L V E S G G G L V K P G G S L R  
 TCTCCTGTTCAGTCTCAGGATTCACCTTCACTAACGCCTGGATGAATTGGGTCCGCCA  
 L S C S V S G F T F T N A W M N W V R Q  
 GGCTCCAGGGAAGGGGCTGGAGTGGGTTGGCCGTATTAAAAACAGAGCTGATGGTGAG  
 A P G K G L E W V G R I K N R A D G E  
 ACAACAGACTACGCTGCACCCGTGAAAGGCCGATTACGCATCTCAAGAGATGAGTCGA  
 T T D Y A A P V K G R F S I S R D E S  
 AAAATATGTTGTTTTTGCAAATGATCAGCCTGAAACCTGAGGACACAGCCATTTATTA  
 K N M L F L Q M I S L K P E D T A I Y Y  
 TTGTACGTCAAATTGTAGTCATAGTCACTGTCCTAAAATTTCTCATATAACGACTAC  
 C T S N C S H S H C P K I S S Y N D Y  
 GATATGGACGTCTGGGGCCCAGGGATCCTGGTACCGTCTCCTCAGGTGGAGGCGGTT  
 D M D V W G P G I L V T V S S G G G G  
 CAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACGTCCTGATGACCCAGTCTCCATC  
 S G G G G S G G G G S D V L M T Q S P S  
 CTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGGGC  
 S L S A S V G D R V T I T C R A S Q G  
 TTTAGAAATGATTTTCGGCTGGTATCAACAGATAACCAGGGAAGCCCCCTAAGTGCCTGA  
 F R N D F G W Y Q Q I P G K A P K C L  
 TCTATGCTGCATCCACTTTGCAGAGTGGGGTCCCATCAAGATTCAGCGGCAGTGGATC  
 I Y A A S T L Q S G V P S R F S G S G S  
 TGGGACAGAATTCATCTCACAAATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTAT  
 G T E F T L T I S S L Q P E D F A T Y  
 TACTGTCTACAACGTAACAATTCCGCCCTCACTTTTCGGCGGAGGGACCAAGGTGGAGA  
 Y C L Q R N N S A L T F G G G T K V E  
 TCAAA  
 I K

**C01a**

CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGG  
Q V Q L V Q S G A E V K K P G A S V K  
TCTCCTGCAAGGCTTCTGGATACACCTTCACCGACTACTATATGAACTGGGTGCGACA  
V S C K A S G Y T F T D Y Y M N W V R Q  
GGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCCTAACAGTGGTGGCACA  
A P G Q G L E W M G W I N P N S G G T  
AAGTATGCACAGAAGTTTCAGGGCTGGGTACCATGACCAGGGACACGTCCATCAGCA  
K Y A Q K F Q G W V T M T R D T S I S  
CAGTCTACATGGAGCTGAGCAGGCTGGGATCTGACGACACGGCCGTGTATTACTGTGC  
T V Y M E L S R L G S D D T A V Y Y C A  
GACAGACCAGATGGCTACAAGTCGAGCGGGGTTTCAGAGGGCTTTGACTTCTGGGGC  
T D Q M A T S R A G V S E G F D F W G  
CAGGGAACCCCTGGTCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTG  
Q G T L V T V S S G G G G S G G G G S  
GCGGTGGCGGATCGGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCC  
G G G G S E I V L T Q S P G T L S L S P  
AGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTA  
G E R A T L S C R A S Q S V S S S Y L  
GCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTACATCCA  
A W Y Q Q K P G Q A P R L L I Y G T S  
GCAGGGCCACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCAC  
S R A T G I P D R F S G S G S G T D F T  
TCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTAT  
L T I S R L E P E D F A V Y Y C Q Q Y  
GCTAGCTCACCTCCCACCTTCGGCCAAGGGACACGACTGGAGATTAAA  
A S S P P T F G Q G T R L E I K

**C01b**

CATGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGG  
H V Q L V Q S G A E V K K P G A S V K  
TCTCCTGCAAGGCTTCTGGATACACCTTCACCGACTACTATATTAAGTGGGTGCGACA  
V S C K A S G Y T F T G Y Y I H W V R Q  
GGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCCTGACAGTGGTGGCACA  
A P G Q G L E W M G W I N P D S G G T  
AAGTATGCACAGAAGTTTCAGGGCTGGGTACCATGACCAGGGACACGTCCATCACCA  
N Y A Q K F Q G W V T M T R D T S I T  
CAGTCTACATGGAGATGAGCAGGCTGGGATCTGACGACACGGCCGTGTATTACTGTGC  
T A Y M E M S R L G S D D T A V Y Y C A  
GACAGACCAGATGGCTACAAGTCGAGCGGGGTTTCAGAGGGCTTTGACTTCTGGGGC  
T D Q M A T S R A G V S E G F D F W G  
CAGGGAACCCCTGGTCACCGTCTCCTCAGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTG  
Q G T L V T V S S G G G G S G G G G S  
GCGGTGGCGGATCGGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCC  
G G G G S E I V L T Q S P G T L S L S P  
AGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTA  
G E R A T L S C R A S Q S V S S S Y L

GCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTACATCCA  
A W Y Q Q K P G Q A P R L L I Y G T S  
GCAGGGCCACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCAC  
S R A T G I P D R F S G S G S G T D F T  
TCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTAT  
L T I S R L E P E D F A V Y Y C Q Q Y  
GCTAGCTCACCTCCCACCTTCGGCCAAGGGACACGACTGGAGATTAAA  
A S S P P T F G Q G T R L E I K



## B – scFv protein data

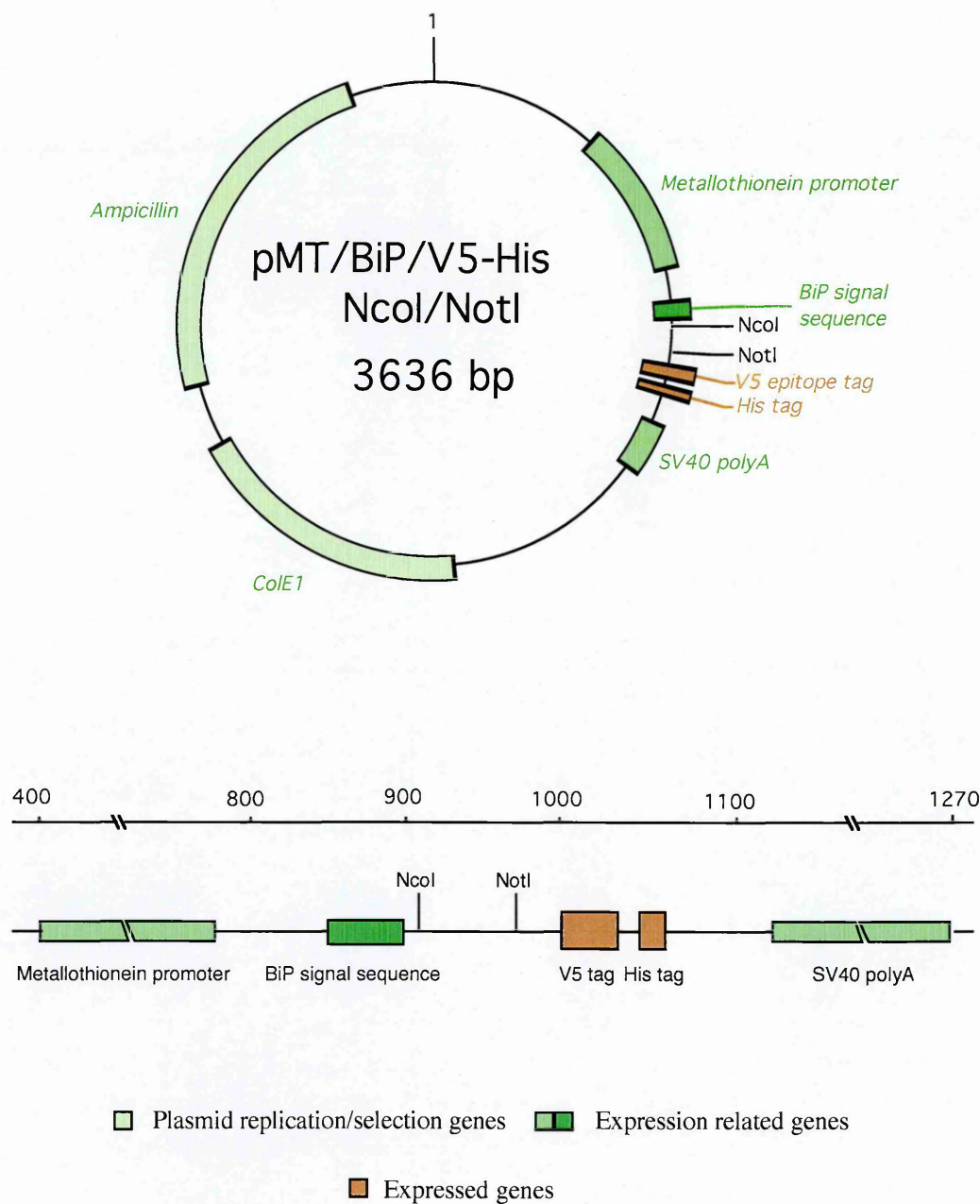
| scFv | Number of amino acids | Estimated M.Wt (kDa) | VH CDR3 length | VL CDR3 length |
|------|-----------------------|----------------------|----------------|----------------|
| B01  | 245                   | 25.1                 | 10             | 11             |
| B02  | 245                   | 26.9                 | 9              | 9              |
| B03  | 246                   | 26.5                 | 15             | 9              |
| B04  | 241                   | 25.8                 | 10             | 8              |
| B05  | 237                   | 25.0                 | 6              | 9              |
| M01  | 243                   | 26.6                 | 10             | 10             |
| M02  | 247                   | 25.8                 | 13             | 11             |
| M03  | 242                   | 25.3                 | 15             | 5              |
| M04  | 239                   | 25.4                 | 9              | 8              |
| M05  | 241                   | 25.7                 | 10             | 9              |
| M06  | 250                   | 26.7                 | 18             | 10             |
| M08  | 243                   | 25.8                 | 12             | 9              |
| S01  | 247                   | 25.8                 | 15             | 10             |
| S02  | 251                   | 27.2                 | 15             | 9              |
| S03  | 241                   | 26.2                 | 10             | 8              |
| S04  | 246                   | 25.9                 | 12             | 11             |
| S05  | 250                   | 26.3                 | 15             | 11             |
| S06  | 253                   | 26.7                 | 22             | 9              |
| S07  | 248                   | 26.5                 | 10             | 9              |
| S09  | 246                   | 26.4                 | 13             | 9              |
| S011 | 263                   | 27.3                 | 28             | 9              |
| S012 | 243                   | 25.9                 | 11             | 10             |
| S013 | 245                   | 25.8                 | 9              | 11             |
| S017 | 244                   | 26.1                 | 7              | 9              |
| S018 | 250                   | 25.9                 | 14             | 9              |
| B2   | 247                   | 26.0                 | 13             | 10             |

### C - Composition of Qiagen buffers used in plasmid DNA purification

| Buffer                                     | Composition   | Storage                          |
|--|---|----------------------------------|
| P1 (resuspension buffer)                   | 50 mM Tris.Cl, pH 8.0<br>10 mM EDTA<br>100 µg/ml RNase A                                | 2-8°C, after addition of RNase A |
| P2 (lysis buffer)                          | 200 mM NaOH<br>1% SDS (w/v)   | 15-25°C                          |
| P3 (neutralization buffer)                 | 3.0 M potassium acetate, pH 5.5   | 15-25°C or 2-8°C                 |
| FWB2 (QIAfilter wash buffer)               | 1 M potassium acetate, pH 5.5   | 15-25°C                          |
| QBT (equilibration buffer)                 | 750 mM NaCl<br>50 mM MOPS, pH 7.0<br>15% isopropanol (v/v)<br>0.15% Triton® X-100 (v/v) | 15-25°C                          |
| QC (wash buffer)                           | 1.0M NaCl<br>50 mM MOPS, pH 7.0<br>15% isopropanol (v/v)                                | 15-25°C                          |
| QF (elution buffer)                        | 1.25 M NaCl<br>50 mM Tris.Cl, pH 8.5<br>15% isopropanol (v/v)                           | 15-25°C                          |
| QG (solubilization buffer)<br>pH indicator | Proprietary information<br>Contains guanidine thiocyanate                               | 15-25°C                          |

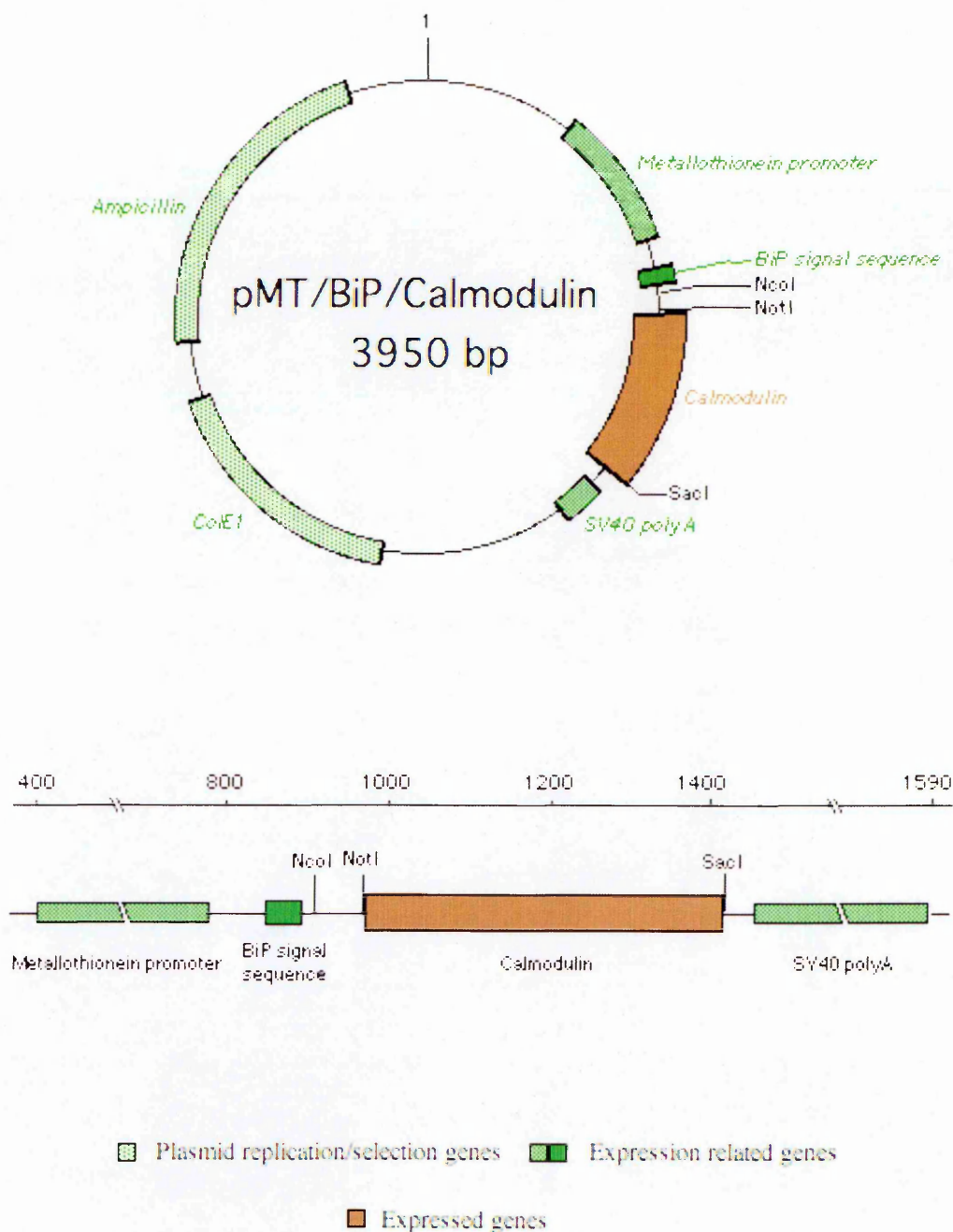
D - Plasmids

D1 – pMT/BiP/V5 His D



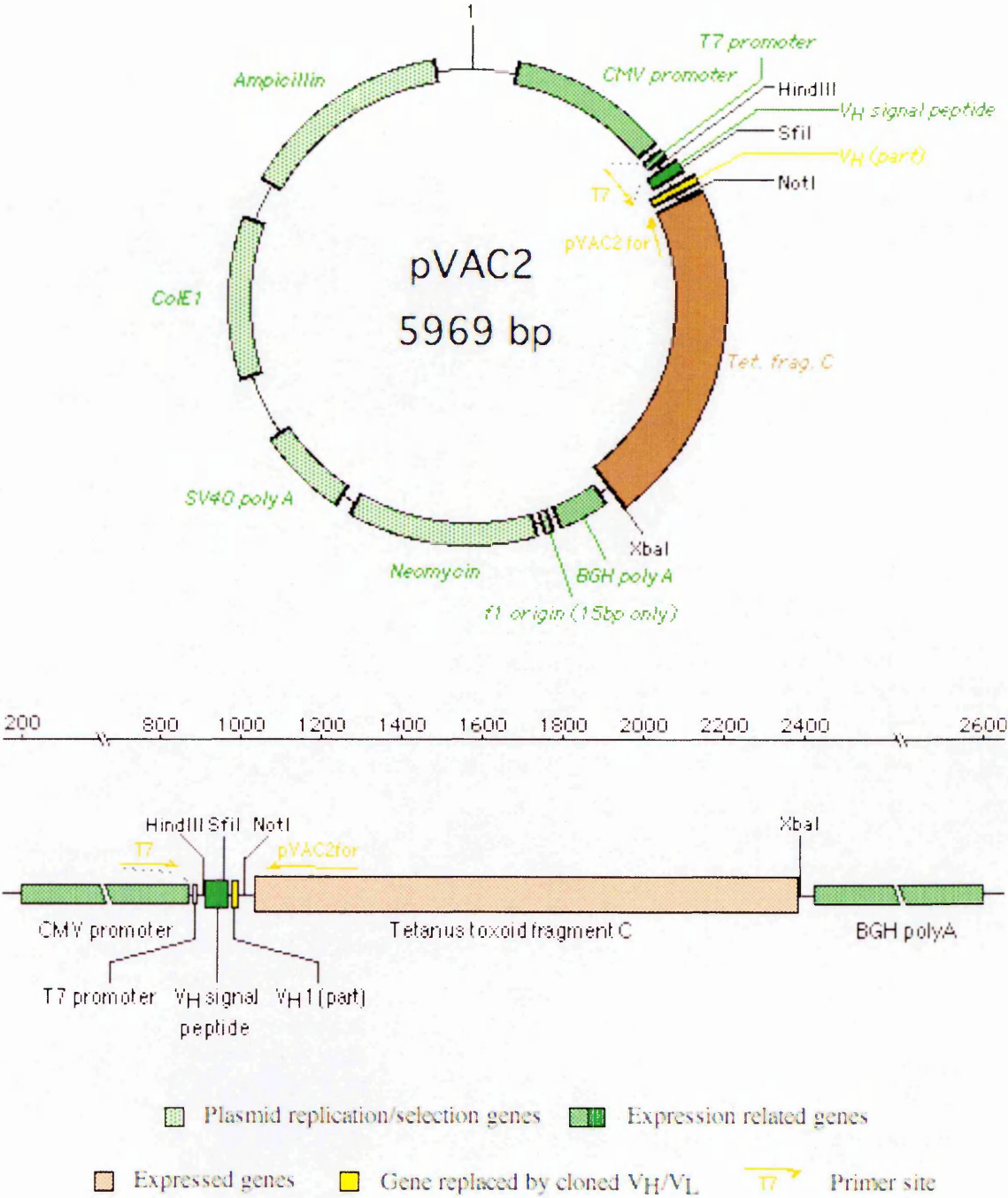
Note: This plasmid is derived from a modified version of pMT/BiP/V5 His D. Two nucleotides were deleted immediately 5' of the NcoI site to make the plasmid compatible with pHenIX, pVAC family, pUC119-His and pUC119-CaM such that scFv cassettes can be cloned in frame with the BiP signal sequence as NcoI/NotI fragments.

D2 – pMT/BiP/CaM

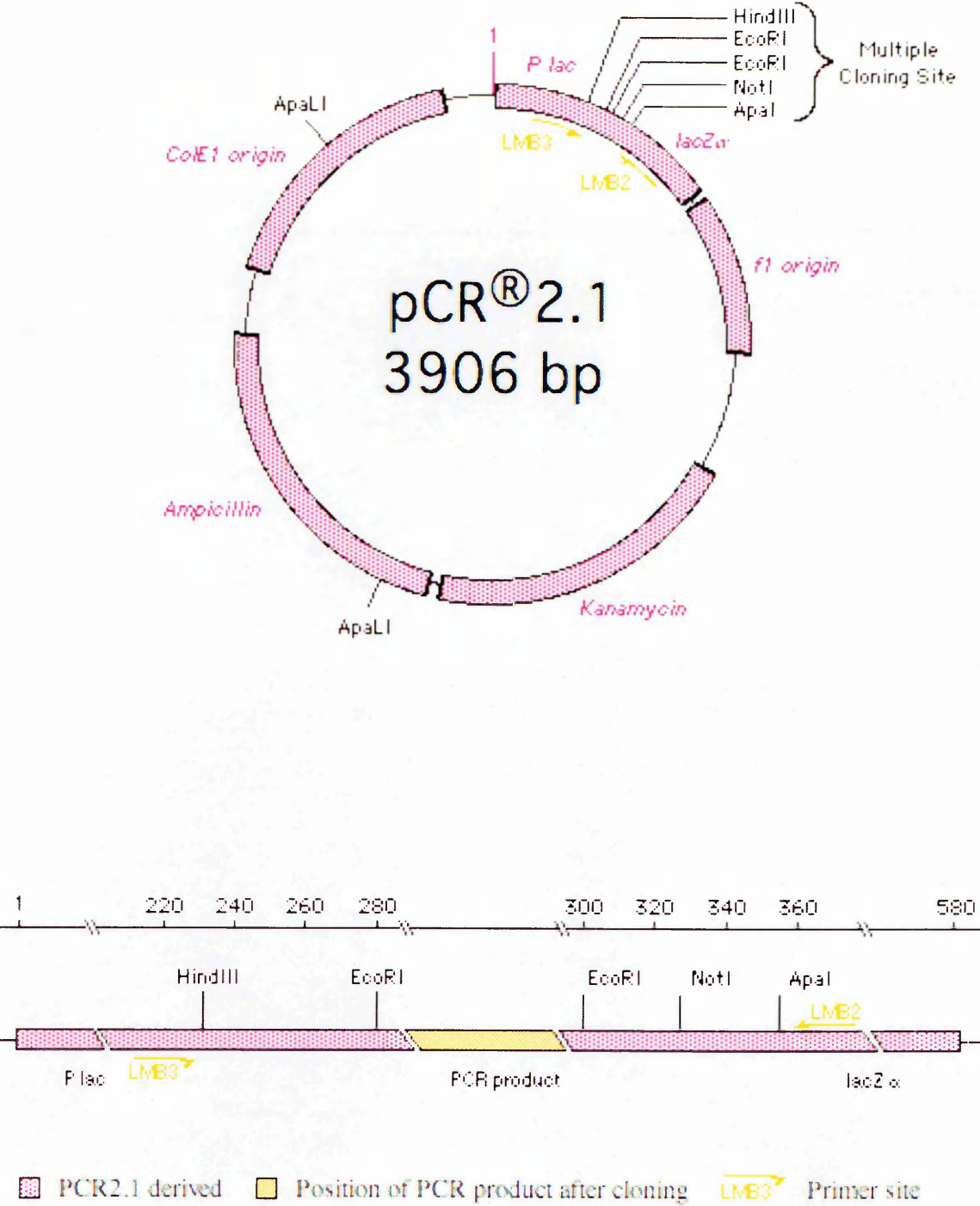


Note: This plasmid is derived from a modified version of pMT/BiP/V5 His D. Two nucleotides were deleted immediately 5' of the NcoI site to make the plasmid compatible with pHenIX, pVAC family, pUC119-His and pUC119-CaM such that scFv cassettes can be cloned in frame with the BiP signal sequence as NcoI/NotI fragments.

D3 – pVAC2



D4 – pCR 2.1 (Invitrogen)



**E - Forms for the preparation of patient DNA vaccines**

**DNA vaccine preparation data sheet 1**

Transfer of scFv cassette from trial centres to Cambridge

Surname:

Forename(s):

Date of Birth:

Study No.:

|  |   |
|--|---|
| From trial centre: .....   |   |
| Date sent: DD/MM/YY  |   |
| Sent as:   | pHenIX-scFv <input type="checkbox"/> pVAC2-scFv <input type="checkbox"/>                  |
| <b>TO BE COMPLETED BY CAMBRIDGE CENTRE</b>   |   |
| Date received: DD/MM/YY  |   |
| Construct documentation <input type="checkbox"/>   | Electronic sequence file <input type="checkbox"/>   |
| Patient status sheet: <input type="checkbox"/>   |   |
| APS/HITS number assigned: <input type="checkbox"/>                                       | <div style="border: 1px solid black; padding: 5px; text-align: center;">Affix Label</div> |
| Print name .....   | Sign name .....   |
| Position .....   |   |
| Comments:  | <div style="border: 1px solid black; height: 50px; width: 100%;"></div>                   |
| Batch of competent bacteria ..... Date transformed: DD/MM/YY                             |   |
| Sequence of clones from stock plate prepared matches reference: <input type="checkbox"/> |   |

DNA vaccine preparation data sheet 2

DNA purification-quality control

Surname:

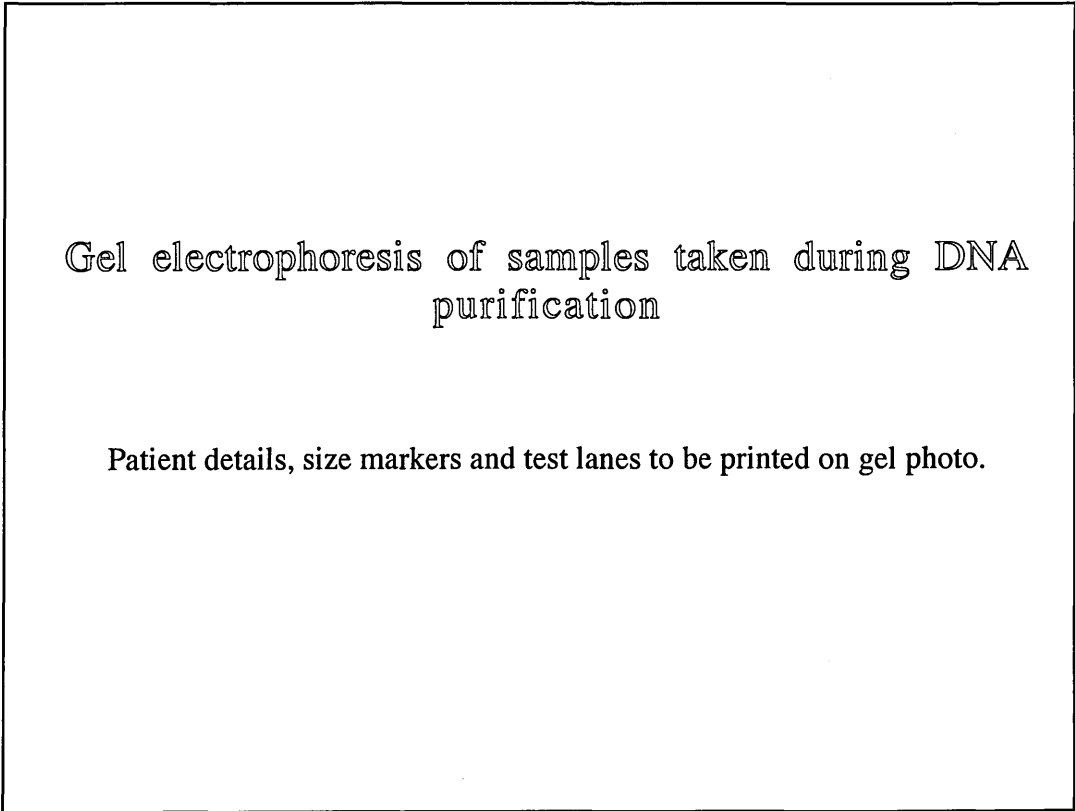
Forename(s):

Date of Birth:

Study No.:

Date of gel:

DD / MM / YY



| TOP ROW            | BOTTOM ROW         |
|--------------------|--------------------|
| 1Kb marker ladder  | 1Kb marker ladder  |
| Lane 1: Sample 1a  | Lane 11: Sample 3a |
| Lane 2: Sample 1b  | Lane 12: Sample 3b |
| Lane 3: Sample 1c  | Lane 13: Sample 3c |
| Lane 4: Sample 1d  | Lane 14: Sample 3d |
| Lane 5: Sample 1e  | Lane 15: Sample 3e |
| Lane 6: Sample 2a  | Lane 16: Sample 4a |
| Lane 7: Sample 2b  | Lane 17: Sample 4b |
| Lane 8: Sample 2c  | Lane 18: Sample 4c |
| Lane 9: Sample 2d  | Lane 19: Sample 4d |
| Lane 10: Sample 2e | Lane 20: Sample 4e |
| 1Kb marker ladder  | Lane 21: Sample 5  |
|                    | 1Kb marker ladder  |



DNA vaccine preparation data sheet 3

DNA purification-quantitation/dilution of final product

Surname: Forename(s):

Date of Birth: Study No.:

Dilution of sample: 1/..... O.D. 260nm =.....

O.D. 280nm =..... Ratio O.D. 260nm : O.D. 280nm =.....

DNA concentration: = O.D.260nm x 50 x Dilution factor = µg/ml

Total quantity of plasmid = .....mg in .....ml

Concentration required for fill: .....µg/ml

Dilution factor from stock to concentration required for fill: 1/.....

.....ml of stock diluted with .....ml of sterile saline to final volume of .....ml

Conformation of concentration of vaccine to be filled:

Dilution of sample: 1/..... O.D. 260nm =.....

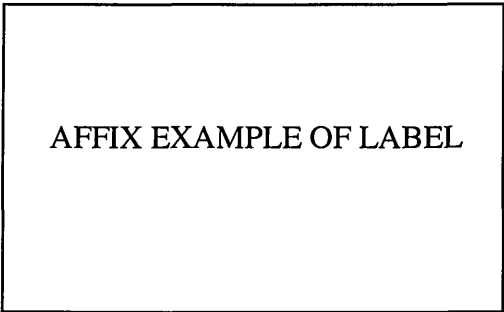
O.D. 280nm =..... Ratio O.D. 260nm : O.D. 280nm =.....

DNA concentration: = O.D.260nm x 50 x Dilution factor = µg/ml

Vial label details and printing

Label details match patient details?

Y / N



DNA vaccine preparation data sheet 4

Vaccine validation - plasmid structure A.

Surname: Forename(s):  
Date of Birth: Study No.:  
Date of digest: DD / MM / YY

Results of SalI digest of pVAC2-scFv  
plasmid

Patient details, size markers and test lanes to be printed on gel photo.

|                                | Expected bands                     |
|--------------------------------|------------------------------------|
| 1Kb marker ladder              |                                    |
| Lane 1: pVAC2 SalI digest      | 2745, 2188, 938, 63 and 34bp.      |
| Lane 2: pVAC2-scFv uncut       |                                    |
| Lane 3: pVAC2-scFv SalI digest | 2745, 2188, 1700 approx. and 34bp. |
| 1Kb marker ladder              |                                    |

Expected band pattern: Y / N

1.5% agarose gel

Comments:

DNA vaccine preparation data sheet 5

Vaccine validation - plasmid structure B

Surname: Forename(s):

Date of Birth: Study No.:

Date of digest: DD / MM / YY

Results of HindIII/NotI and SfiI/NotI digests of  
pVAC2-scFv plasmid

Patient details, size markers and test lanes to be printed on gel photo.

|  | Expected bands           |
|--|--------------------------|
| 1Kb marker ladder                      |                          |
| Lane 1: pVAC2-scFv uncut               | N/A                      |
| Lane 2: pVAC2-scFv HindIII/NotI digest | Approx. 900bp and 4552bp |
| Lane 3: pVAC2-scFv SfiI/NotI digest    | Approx. 850bp and 4606bp |
| 1Kb marker ladder                      |                          |

Comments:

DNA vaccine preparation data sheet 6

Vaccine validation – Endotoxin/protein levels and microbiology

Surname: Forename(s):

Date of Birth: Study No.:

Endotoxin level: .....IU/ml

OD 260/280 ratio: ..... <2.0 = good 1.1-2.0 = acceptable

Microbiology: STERILE / NON-STERILE

DNA vaccine preparation data sheet 7

Final approval:

Surname:

Forename(s):

Date of Birth:

Study No.:

Plasmid structure - digests:

HindIII/NotI ☐ SalI ☐ NotI/SfiI ☐

Plasmid structure - scFv sequence matches reference: ☐

Microbiology: ☐

Endotoxin level: ☐ OD 260/280 ratio: ☐

DNA concentration after sterile fill: .....µg/ml

Print name..... Sign ..... Research Assistant

Print name..... Sign ..... Research Associate

Print name..... Sign ..... Principal Investigator

## DNA vaccine preparation data sheet 8

### Batch summary:

Surname:

Forename(s):

Date of Birth:

Study No.:

Quantity of plasmid purified: ..... mg in .....ml

Quantity diluted for sterile fill: ..... mg

Number of vials filled: ..... of ..... ml at .....µg/ml

Number of vials sent for endotoxin testing: .....

Number of vials sent for microbiology testing: .....

Number of vials sent for protein testing: .....

Number of vials issued for vaccination:

..... Date: DD / MM / YY

..... Date: DD / MM / YY

..... Date: DD / MM / YY

..... Date: DD / MM / YY

..... Date: DD / MM / YY

..... Date: DD / MM / YY

Number of vials used for research:

..... Date: DD / MM / YY

..... Date: DD / MM / YY

Number of vials remaining in storage at end of trial: .....

Signed: .....

Name: .....

DNA vaccine preparation data sheet 9

Sterile fill production record part 1 – process data

Surname: Forename(s):  
Date of Birth: Study No.:

Product volume: .....ml Processing date: DD / MM / YY

Serology: Known ☐ No result ☐

Start time: ..... Finish time: .....

Logged to freezer at: .....

No. of labels issued: ☐ destroyed: ☐

Affix label here

Filter pressure test:

- |    |             |    |             |
|----|-------------|----|-------------|
| 1) | PASS / FAIL | 2) | PASS / FAIL |
| 3) | PASS / FAIL | 4) | PASS / FAIL |
| 5) | PASS / FAIL | 6) | PASS / FAIL |
| 7) | PASS / FAIL | 8) | PASS / FAIL |

No. product vials filled: ☐

Signed: ..... Name: .....

DNA vaccine preparation data sheet 10

Sterile fill production record part 2 – disposables record

Surname: Forename(s):

Date of Birth: Study No.:

Processing date: DD / MM / YY

|          | Manufacturer | Lot # | Sterilisation Run # |
|----------|--------------|-------|---------------------|
| Vials    | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
| Syringes | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
| Needles  | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
| Filters  | .....        | ..... | .....               |
|          | .....        | ..... | .....               |
| TS broth | .....        | ..... | .....               |
|          | .....        | ..... | .....               |

Signed: ..... Name: .....



DNA vaccine preparation data sheet 11

Patient status:

Surname:

Forename(s):

Date of Birth:

Study No.:

|                                       |                                  |                                  |
|---------------------------------------|----------------------------------|----------------------------------|
| WHO performance status:               | <input type="text" value="≤2"/>  | <input type="text" value="≥2"/>  |
| Platelet count >100x10 <sup>9</sup> L | <input type="text" value="Yes"/> | <input type="text" value="No"/>  |
| M band on serum electrophoresis       | <input type="text" value="No"/>  | <input type="text" value="Yes"/> |
| Anti-DNA antibodies:                  | <input type="text" value="No"/>  | <input type="text" value="Yes"/> |
| Rheumatoid factors:                   | <input type="text" value="No"/>  | <input type="text" value="Yes"/> |
| Anti-muscle antibodies:               | <input type="text" value="No"/>  | <input type="text" value="Yes"/> |

|   |  |                                  |
|---|--|----------------------------------|
| INFECTIOUS DISEASE SCREEN. TO BE COMPLETED BY<br>CAMBRIDGE CENTRE |  |                                  |
| Date sample for screening taken                                   | <input type="text" value="DD / MM /..YY"/> |                                  |
| Anti-HBs antibodies:  | <input type="text" value="No"/>            | <input type="text" value="Yes"/> |
| Anti-HBc antibodies:  | <input type="text" value="No"/>            | <input type="text" value="Yes"/> |
| Anti-HCV antibodies:  | <input type="text" value="No"/>            | <input type="text" value="Yes"/> |
| Anti-HIV1/2 antibodies:   | <input type="text" value="No"/>            | <input type="text" value="Yes"/> |
| TPHA negative:  | <input type="text" value="Yes"/>           | <input type="text" value="No"/>  |